Molecular Typing and Analysis of Antimicrobial Resistance in Clinical Isolates of *Streptococcus pneumoniae* and *Haemophilus influenzae*

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ABSTRACT

β-Lactams are widely used to treat *S. pneumoniae* and *H. influenzae* respiratory infections. However, resistance to β-lactams is increasing in the UK. To establish if this results from spread of a small number of resistant strains or from a wider emergence of resistance, the molecular type and mechanisms of resistance in *S. pneumoniae* and *H. influenzae* isolates, from throughout the UK, were determined.

Resistance of *S. pneumoniae* to penicillin is due entirely to the development of altered forms of the high molecular weight penicillin binding proteins (PBPs) and decreased affinity of PBP 2x, 2b and 1a together give high-level penicillin resistance. Between 1995 and 1996, 80 isolates of *S. pneumoniae* were obtained from centres throughout the UK. Eighteen isolates were penicillin-resistant and these were typed by pulsed-field gel electrophoresis (PFGE). In addition, changes in penicillin binding proteins (PBPs) 2x, 2b and 1a were examined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that penicillin resistance was not simply the result of clonal spread. Screening for changes in PBPs and RFLP of the amplified *pbp2x, 2b* and *1a* genes by *Hin*fI showed that there was a correlation between resistance and alteration in PBPs. The results indicate that penicillin resistance in *S. pneumoniae* in the UK is evolving in a variety of different strains and this raises cause for concern.

β-Lactam resistance in *H. influenzae* is mainly mediated by the production of β-lactamases. β-lactamase-mediated resistance is associated with the production of TEM-1, ROB-1 and VAT-1 β-lactamase enzymes. Impermeability and alteration in PBPs are further mechanisms that mediate resistance to β-lactams. The epidemiology and mechanisms of resistance to amoxycillin and amoxycillin/clavulanic acid (co-amoxiclav) in *H. influenzae* isolates from the UK were examined. Between 1995 and 1996, 231 isolates

of *H. influenzae* were obtained from centres throughout the UK. Twenty percent of isolates were amoxycillin-resistant and, of these, 19% were also resistant to co-amoxiclav. Amoxycillin-resistant isolates were genotyped by PFGE, and screened for β-lactamase production by isoelectric focusing (IEF) followed by PCR employing primers for the TEM-1 β-lactamase. IEF demonstrated that all the β-lactamase-positive strains produced an enzyme that co-focused with TEM-1. PCR confirmed the identity of the β-lactamase. Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that amoxycillin-resistant isolates susceptible to clavulanic acid showed little genetic relatedness. In contrast, among co-amoxiclav-resistant isolates only two major groups of strains were identified. One group were TEM-1 producers and exhibited high MICs of amoxycillin (>8 mg/L) which were reduced by the addition of clavulanic acid. The second group had the same MIC of amoxycillin as that of co-amoxiclav and showed no evidence of β-lactamase production. Impermeability and alterations in PBPs were examined in these isolates. Amoxycillin resistance in *H. influenzae* throughout the UK is mediated by the TEM-1 enzyme which appears to have spread into a wide variety of different strains. In contrast emerging resistance to clavulanic acid appears to be restricted to a few strains.

DECLARATION

The experiments and composition of this thesis are the work of the author unless otherwise stated

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ABBREVIATIONS

7-ACA	7-aminocephalosporanic acid
AMP^r β^-	Ampicillin-resistant non-β-lactamase-producing strain
6-APA	6-aminopenicillanic acid
ATP	adenosine triphosphate
BHI	brain-heart infusion
bla	β-lactamase gene
bp	base pair
BSAC	British Society for Antimicrobial Chemotherapy
cfu	colony forming unit
CSF	cerebrospinal fluid
DHP-I	dehydropeptidase I
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EL	EDTA-lauroylsarcosine
GyrA	DNA gyrase A subunit
GyrB	DNA gyrase B subunit
h	hour
Hib	*H. influenzae* serotype b
HPLC	high-pressure liquid chromatography
IEF	isoelectric focusing
kbp	kilobase pairs
KDa	kiloDalton
LPS	lipopolysaccharide
MIC	minimum inhibitory concentration
MIC_{50}	MIC at which 50% of the tested isolates were found to be susceptible
MIC_{90}	MIC at which 90% of the tested isolates were found to be susceptible

min	minutes
MLEE	multilocus enzyme electrophoresis
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NCCLS	National Committee for Clinical Laboratory Standards
NCHI	non-capsulated *H. influenzae*
NCTC	national collections of type cultures
nm	nanometers
OD	optical density
OMP	outer-membrane protein
PAGE	polyacrylamide gel electrophoresis
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
pI	isoelectric point
PMSF	phenylmethylsulphonyl fluoride
QRDR	quinolone resistance-determining region
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
TMP-SMZ	trimethoprim-sulfamethoxazole
TN	Tris-NaCl
Tris	tris (hydroxymethyl) methylamide
U	units of enzyme activity

UPGMA	unweighted pair group method with arithmetic averages
UV	ultra-violet light
V	voltage
W	watt

Single-letter amino acid codes

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	aspargine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

PRESENTATIONS

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CHAPTER ONE

Introduction

1.1. Bacterial Respiratory Tract Infections

1.1.1. Causative Organisms

Bacterial respiratory tract infections are a serious health problem worldwide. These infections are very prevalent and continue to be a leading cause of morbidity and mortality (Klugman, 1990). These airway infections often involve more than a single bacterial pathogen and interactions between bacteria can worsen the problems of bacterial resistance, so choosing the most appropriate antibiotic remains a key clinical decision.

Although several microorganisms can produce respiratory tract infections, the most common bacterial pathogens are *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis*, as well as *Mycobacterium tuberculosis.*

Staphylococcus aureus and gram-negative bacilli, though rare causes of community-acquired pneumonia are, however, associated with the highest rates of mortality, 39 & 33%, respectively (Gilbert & Fine, 1994). Mortality associated with *S. pneumoniae* and *H. influenzae* is much lower, at about 11 and 2%, respectively (Gilbert & Fine, 1994). Atypical bacterial pathogens, such as *Mycoplasma pneumoniae* and *Legionella* spp. are not sufficiently common to allow an accurate determination of mortality.

For the purpose of this thesis, almost all my writing will be to discuss in details the two most commonly isolated pathogens in airway infections: *S. pneumoniae* and *H. influenzae.*

1.1.2. Upper Airway Infections

i. Acute & Chronic Otitis Media

This condition is very common in infants and small children, partly because the auditory tube is open more widely and immunity is lower at this age. One study showed that 80-90% of children have at least one episode before they are 2 years old (Teele *et al.*, 1989).

H. influenzae is the cause of 10-30% of acute otitis media whereas *S. pneumoniae* accounts for 20-40% of the cases (Martin *et al.*, 1995). In chronic otitis media, *H. influenzae* predominates (15 vs. 7% with *S. pneumoniae*). More than 90% of *H. influenzae* responsible are non-capsulated (Klein, 1990) and only a minor percentage are *H. influenzae* type b. *S. pneumoniae* causes otitis media in children, affecting up to 7 million American children a year (MMWR, 1996).

ii. Sinusitis

Sinusitis can occur at all ages and it develops as a complication in 1-5% of all upper airway infections. Although sinusitis is a common infection, it is potentially dangerous because of the proximity to the central nervous system. This condition develops when allergy or viral infections obstruct normal drainage and aeration. In the first stages of the infection the most common pathogens are *S. pneumoniae* and *H. influenzae*. Anaerobic organisms become involved as the infection turns chronic and the levels of tissue oxygen decline (Carenfelt & Lundberg, 1977).

iii. Epiglottitis

H. influenzae type b is one of the most common cause of epiglottitis (Albritton, 1988). Although the incidence of *H. influenzae* epiglottitis is high in children, its occurrence in adults is also well recognised (Frantz & Rasgon, 1993). However, cases of epiglottitis in children appear to be diminished since the introduction of *H. influenzae* type b vaccine (Takala *et al.*, 1994).

1.1.3. Lower Airway Infections

Lower airway infections are a very common cause of morbidity and mortality.

i. Acute Bronchitis

This is an inflammatory condition of the tracheobronchial tree usually associated with viral infection. Secondary bacterial infection with *S. pneumoniae* and *H. influenzae* may play a role in pathogenesis.

ii. Acute Exacerbations of Chronic Bronchitis

Bacterial infection does not appear to initiate the disease but is probably significant in perpetuating it and in producing the characteristic acute exacerbations. Infection appears to be only one component of the syndrome, the other being cigarette smoking and inhalation of the dust or fumes from the workplace (Brook, 1994). *S. pneumoniae* and non-capsulated strains of *H. influenzae* are the organisms most frequently isolated.

iii. Community-Acquired Pneumonia

The classical bacterial cause of community-acquired pneumonia is *S. pneumoniae* with *H. influenzae* the second most common bacterial pathogen. *S pneumoniae* also seems to be the most frequent cause of severe community-acquired pneumonia (Moine *et al.*, 1994). In the UK, *S. pneumoniae* is responsible for 30-50% of community-acquired pneumonia (Meyer & Finch, 1992). Though the case fatality rate in pneumococcal pneumonia decreased significantly with the advent of antibiotics in the 1940s, the incidence has remained almost unchanged (Austrian, 1981). Interestingly, the frequency of pharyngeal carriage has fallen dramatically, but the incidence of pneumococcal infection remains unchanged (Foy *et al.*, 1975).

1.1.4. Infections in Immunocomprised Patients

Infection is a frequent complication of patients with the acquired immune deficiency syndrome (AIDS). Approximately 5% of all hospital admissions of AIDS patients are due to bacterial pneumonia. Pneumococcal pneumonia may be the first manifestation of human immunodeficiency virus (HIV) infection in a substantial number of patients (Garcia-Leoni *et al.,* 1992). Also *S. pneumoniae* is the leading cause of invasive bacterial respiratory disease in adults and children with HIV infection (Janoff *et al.,* 1992).

1.1.5. Nosocomial Infections

Nosocomial infections are an important cause of morbidity and mortality. It has been estimated that approximately 5% of patients in the United States develop a hospital-acquired infection (Haley *et al.,* 1985). A recent report on pathogens reported from nosocomial infections in hospitals in the United States found *S. pneumoniae* to be the fourth most frequent pathogen causing nosocomial pneumonia (accounting for 6.5% of the infections) (Jarvis & Martone, 1992).

1.1.6. Invasive Infections

H. influenzae used to be the most common cause of bacteraemic disease and meningitis in children with the great majority of cases caused by encapsulated strains of type b (Moxon & Wilson, 1991); however the introduction of a vaccine against type b strains has altered this (Slack, 1995). *S. pneumoniae* is one of the leading causes of acute bacterial meningitis with bacteraemia. In the United States the overall incidence of pneumococcal meningitis is 1.1 per 100 000 population, but in infant under 5 months old the incidence is higher at 30 in 100 000 with 10% mortality (Schelch *et al.,* 1985).

Many underlying conditions are important for susceptibility to pneumococcal infections. *S. pneumoniae* is responsible for at least half of the cases of infection with encapsulated bacteria in asplenic individuals and for most of the fatal ones (Kalin,

1998).. The reason that the splenectomised individuals are more susceptible to infection with encapsulated bacteria is probably the spleen's unique role in the clearance of non-opsonised organisms (Bruyn *et al.*, 1992).

1.1.7. Epidemiological Aspects

i. Healthy Carrier

S. pneumoniae is an exclusively human pathogen, which is spread from person to person by aerosols. It is carried in the nasopharynx of up to one third of the adult population without any apparent symptoms, and this is presumably how it manages to persist as a human parasite.

Most people harbor *H. influenzae* in their pharynx and, to a lesser extent, these organisms also colonise the mucosa of the conjunctiva as well as the genital tract (Moxon, 1990). Although respiratory carriage of non-capsulated organisms is common (30-80%), the carriage rate of the *H. influenzae* type b is much lower (2-5%).

ii. Seasonal Occurrence

Although studies by Davies and Maesen (1986) showed no constant seasonal variation in infections caused by *H. influenzae*, seasonal variation was observed by Howard *et al.*, (1991) for invasive infections caused by type b strains of *H. influenzae*; infections arose more commonly in the winter months. In *S. pneumoniae* there is seasonal variation in the incidence of pneumococcal infections. In temperate climates infections are most frequent in winter but in the tropics they occur mostly at the end of the dry season (Klein, 1982).

1.2. *Streptococcus pneumoniae*

1.2.1. Historical Perspective

S. pneumoniae has been the subject of intensive investigation at both the clinical and basic scientific level during the past century. In a number of instances, these studies have resulted in important progress toward the comprehension of basic biological principles (Watson *et al.*, 1993).

S. pneumoniae was first visualised in pulmonary tissues by Klebs in 1875, later by Eberth in 1880, and then by Koch in 1881 (Reviewed by Austrian, 1981). However, the organism was first isolated in the laboratory, from human saliva, independently by George Sternberg in the United States and Louis Pasteur in France, and reported by both in 1881 (Reviewed by Austrian, 1981). At the turn of the century, Neufeld reported on the solubility of pneumococci in bile (Reviewed by Austrian, 1981). Most important were demonstrations of the development of antibodies against the homologous pneumococcus following infection and vaccination and the description of the specific agglutination and Quellung reactions in homologous antisera by Neufeld in 1902 (Reviewed by Austrian, 1981). In 1928, Griffith demonstrated that the genetic material of the virulent *S. pneumoniae* strain, which remained intact despite being heated, was released from the heat-killed cells and was able to transform a non-virulent strain into a virulent one (Griffith, 1928). This experiment led to the principle of the genetic transformation.

1.2.2. Morphology & Growth Characteristics

S. pneumoniae is a gram-positive coccus, about 1.0 μm in diameter, and may be ovoid or lanceolate, occurring in pairs with the broader ends opposed. It is a relatively fastidious organism that grows best on complex media containing additives, such as blood or serum, and is classified as an aerobe and facultative anaerobe, but most strains prefer an atmosphere of 5-10% CO_2 for primary culture. On culture pneumococci are α-haemolytic but are distinguished from other α-haemolytic

streptococci by optochin sensitivity and by sensitivity to surface-active agents such as ox bile or sodium desoxycholate.

1.2.3. Capsular Serotypes

The capsules of *S. pneumoniae* are made up of polysaccharides varying in their mono- and disaccharide components (Lund & Henrichsen, 1978). There are 90 different capsular serotypes together with their antigenic formulae presently known (Henrichsen, 1995). Two serotyping nomenclatures of pneumococcal serotypes exist, namely the Danish and the American systems, but the consensus usage has for some time been the Danish system, with all typing sera provided by the Statens Serum Institute, Copenhagen (Lund & Henrichsen, 1978). Serotyping is based on the structural diversity of the capsular polysaccharide. The first stage of serotyping involves the use of pools of sera, and then individual sera from positive pool, to assign a number, such as 1, 3, and 14. No further subtyping is possible and hence the number is referred to as a serotype. For others, such as 6, 19, and 23, subtyping sera can distinguish further subtypes such as 6A and 6B, 19F (First), 19A, 19B and 19C, and so on; in these cases the number is referred to as the serogroup, and the number plus letter as the serotype or subtype. This nomenclature reflects the cross reactivity of subtypes within a serogroup (Lund & Henrichsen, 1978).

The prevalence of these serotypes varies around the world and amongst different age groups, with certain capsular serotypes being more or less effective immunogens than others. It is not clear which factors influence the worldwide distribution of serotypes. However, there could be a selective advantage for pneumococci to switch serotypes as they move around the population, perhaps to avoid clearance during carriage, or to further evade the immune system during disease (Dowson *et al.*, 1997).

1.2.4. Pathogenicity

S. pneumoniae is carried in the upper respiratory tract by many healthy individuals. The mechanisms by which pneumococci translocate from the nasopharynx to the lung, thereby causing pneumonia, or migrate directly to the blood, giving rise to bacteraemia or septicemia, are poorly understood (AlonsoDeVelasco *et al.*, 1995). Most infections do not occur after prolonged carriage but follow the acquisition of recently acquired serotypes (Johnston, 1991). This suggests that the immune status of the host at the moment of colonisation, as well as the virulence of the particular strain, determines whether pneumococci will remain confined to the nasopharynx or become invasive.

Unrestrained multiplication of pneumococci in the lungs, meninges, or middle ear will result in pneumococcal lysis. There is a release of lysozyme in the mammalian secretion fluids at the sites of infection may contribute to pneumococcal lysis through the activation of autolysin (Cottagnoud & Tomasz, 1993). Pneumococcal lysis will, in turn, trigger the inflammatory process, directly by attracting and activating phagocytes and indirectly through complement activation and toxin formation. There is increasing support for the hypothesis that such inflammation may be responsible for the morbidity and mortality of pneumococcal infection (Musher, 1992).

1.2.5. Virulence Factors

i. Capsule Polysaccharide

It has been known since the beginning of the century that the polysaccharide capsule is crucial for the virulence of the pneumococcus (Johnston, 1991). Rough strains without capsules are virtually avirulent, the number of pneumococci that are required to establish a lethal infection in laboratory animals being increased by a factor of 10^5. Though necessary for virulence, the capsular polysaccharides are non-toxic when injected into laboratory animals as pure substances. The mechanism by which they promote virulence is by protecting the bacteria against immediate ingestion by animal phagocytes. However, there is a considerable difference in virulence among the 90

known capsular types; some of the capsular structures are connected with significant invasiveness (e.g., 6, 9, 19, 23) while most of them seem to confer only minor virulence.

ii. Cell Wall & Cell Wall Polysaccharide

Toxic products produced during growth or degradation have been thought to be responsible for the often dramatic clinical presentation of a pneumococcal infection, but no single toxin or other cell product has been definitively identified as responsible. However, cell wall constituents seem to be largely responsible for the inflammatory reaction (Tuomanen *et al.,* 1985; Boulnois, 1992). The phosphorylcholine residue of the C-polysaccharide is highly immunogenic, eliciting antibody response, complement activation and binding of an acute phase reactant β-globulin of human serum, the C reactive protein. Furthermore, the latter reaction results in opsonisation and activation of complement.

iii. Pneumococcal Protein Antigens & Toxins

Various proteins have been suggested to be involved in the pathogenicity of *S. pneumoniae*. Two enzymes, neuraminidase and pneumolysin, released primarily during autolysis, may be of importance in the pathogenesis (Bruyn *et al.,* 1992; Boulnois, 1992). The mechanism of release may be the reason why autolysin-deficient strains are less virulent than autolysin-producing variant. Also, the surface protein A may be involved in the pathogenetic events during pneumococcal infection (Bruyn *et al.,* 1992; Boulnois, 1992). Both pneumolysin- and protein A-deficient strains show reduced virulence in experimental animals with prolonged survival of the animals after pneumococcal challenge. The same effect can also be achieved by active or passive immunisation of the animal against either of these two antigens. Pneumolysin also seems to be directly injurious to the pulmonary vascular endothelium (Rubins *et al.,* 1993). However, although several new pathogenic facets have emerged in recent years, the importance of different mechanisms by which pneumococcal cell wall or other constituents, alone or in concert with each other and with host factors, damage the host are still largely obscure.

1.3. *Haemophilus influenzae*

1.3.1. Historical Perspective

In 1892, Pfeiffer claimed that the small gram-negative haemophilic coccobacillus that he had isolated in large numbers from the sputum of patients suffering from epidemic influenza was the causative agent of that disease (Slack & Jordens, 1998). The specific name *H. influenzae* (Winslow *et al.*, 1917) which was given to the organism is a permanent reminder of this erroneous association.

1.3.2. Morphology & Growth Characteristics

H. influenzae is a slender, short, poorly staining gram-negative rod or coccobacillus, 0.3-0.5 μm x 0.5-1.0 μm with rounded ends. Specific growth factor requirements are major criteria by which the genus *Haemophilus* is defined. The species will not grow in the absence of certain factors that are present in blood, hence the generic name *Haemophilus* or 'blood-loving'. *H. influenzae* requires 2 accessory growth factors, a heat-stable growth promoting substance present in blood cells (X factor), and a heat-labile vitamin-like substance (V factor). X factor is involved in oxidation-reduction processes in the growing bacterial cell and it can be satisfied by iron-containing compounds like haems. V factor is essential for the synthesis of iron-containing respiratory enzymes and it can be satisfied by nicotinamide-adenine dinucleotide (NAD^+) or NAD phosphate ($NADP^+$). The optimal medium for growth is chocolate-blood agar at 35-37° in 5-10% CO_2.

1.3.3. Capsular Serotypes

In 1931 Pittman found that *H. influenzae* strains isolated from the cerebrospinal fluid or blood of patients with meningitis appeared iridescent when cultured on Levinthal's agar. This iridescence was shown to be caused by the presence of a polysaccharide capsule (Pittman, 1931). Subsequently, six serotypes (a-f) based on capsular antigen were described. Each capsule is composed of a linear polymer of disaccharide units. The capsules are negatively charged, acidic and hydrophobic. Types a and b differ from the others in containing the 5-carbon component ribitol, and type b is unique as

both sugars are pentoses. However, not all organisms express this surface antigen; those that do not are referred as non-capsulate *H. influenzae* (NCHI).

1.3.4. Classification of Biotypes

In 1976 Kilian developed a biochemical scheme for classifying *H. influenzae* into eight biotypes, namely biotypes I-VIII, based on 3 biochemical tests (indole, urease and ornithine decarboxylase) (Kilian, 1976 & 1985). Most clinical isolates of *H. influenzae* are distributed between 4 biotypes (I, II, III & IV); *H. influenzae* type b isolates from invasive disease are mainly biotype I and the majority of NCHI are biotype II or III (van Alphen, 1993).

1.3.5. Pathogenicity

i. Capsulate *H. influenzae*

H. influenzae is the most important species causing human disease. It is strictly a parasite of Man and is associated with the normal commensal flora of the respiratory tract. Infections caused by *H. influenzae* generally result from invasion of the bloodstream or contiguous spread from the respiratory tract. It causes serious invasive disease in children under 5 years of age, such as meningitis. Up to 20% of survivors of *H. influenzae* type b meningitis have long term neurological sequelae. Risk factors for *H. influenzae* type b disease are mainly socioeconomic and include overcrowding, attendance at day-care centres, chronic illness and lack of access to good health care facilities (Jorden & Slack, 1995).

ii. Non-Capsulate *H. influenzae* (NCHI)

NCHI are often associated with infection of previously damaged tissues, causing respiratory infections in patients with chronic bronchitis, bronchiectasis or cystic fibrosis. Recent reports show that NCHI are also an important cause of invasive disease in both adults and children (Jorden & Slack, 1995). In most cases there are predisposing factors such as head injury, Otitis media or sinusitis, and the infection is presumed to result from direct extension (Bol *et al.*, 1987).

1.3.6. Virulence Factors

i. Capsulate *H. influenzae*

The possession of the type b capsule has been shown to be a major virulence factor. The bacterial capsule aids colonisation and is probably the most important virulence factor for invasive disease as it protect against phagocytosis and complement-mediated lysis (Tunkel & Scheld, 1993). Other virulence factors which may be involved in adherence and colonisation of *H. influenzae* type b include lipopolysaccharide (LPS) (Moxon & Maskell, 1992), which is expressed differently in the nasopharynx and the bloodstream, and fimbriae, which have been shown to be important for nasopharyngeal colonisation but not for invasion (van Alphen & van Ham, 1994). Outer-membrane protein P2 (OMP P2) has been shown to affect the virulence of *H. influenzae* type b (Sanderes *et al.,* 1993) and peptidoglycan was shown to cause brain oedema and inflammation by increasing blood-brain barrier permeability (Burroughs *et al.,* 1993). Haemocin, a bacteriocin, is strongly associated with type b strains (LiPuma *et al.,* 1992). IgA proteases are produced by all *H. influenzae* type b but have been shown to be inhibited by human milk (Plaut *et al.,* 1992).

ii. Non-Capsulate *H. influenzae* (NCHI)

Recent studies suggest that fimbriae (Sirakova *et al.,* 1994) and OMP P2 may be important virulence factors for NCHI (Sanders *et al.,* 1993). The secretory products IgA protease and ciliotoxin may contribute to the pathogenesis of pneumonia (Moxon & Wilson, 1991). Transferrin-binding proteins have been detected on NCHI (Hardie *et al.,* 1993). Peptidoglycan from NCHI caused inflammation of the middle ear (Leake *et al.,* 1994).

1.4. Prevention-1

Immunisation

Pneumococcal vaccines based on the capsular polysaccharides from the 23 serotypes most commonly associated with disease have been available for some years (Klein, 1999). These have serious limitations as they fail to protect young children and do not elicit immunological memory. Invasive *H. influenzae* disease has been essentially eliminated among vaccinated children and, since the vaccine reduces the level of carriage, which decreases transmission within the community (Booy & Kroll, 1994, Slack, 1995). The success of the *H. influenzae* serotype b vaccine has depended on special circumstances that do not apply to the pneumococcus. Firstly, almost all invasive disease is caused by serotype b isolates of *H. influenzae*, whereas a substantial proportion of the >90 serotypes of *S. pneumoniae* can cause serious disease. Secondly, there is clear evidence that pneumococci can relatively frequently change the polysaccharide capsule that they express, which may lead to vaccine escape (Crook & Spratt, 1998), whereas alterations of serotype are extremely rare in *H. influenzae*. These deficiencies in pneumococcal vaccine are being addressed by the introduction of vaccines in which the capsular polysaccharides from about eight of the most important serotypes are conjugated to a protein carrier and a number of these conjugate vaccines are in clinical trials (Kayhty & Eskola, 1996, Klein, 1999).

1.5. Treatment of Infections

i. Treatment of *Streptococcus pneumoniae* Infections

Since it became available for clinical use in the early 1940s, penicillin has been the antibiotic of choice for treating pneumococcal infections, so causing a marked reduction in mortality and morbidity. The efficacy of penicillin for the treatment of pneumococcal infections was unquestioned until reports of strains resistant to penicillin began to emerge (Klugman, 1990). The earliest reports of such resistance came from an area in Papua New Guinea where systemic prophylaxis with penicillin had been tested as an approach to the control of pneumococcal pneumonia (Klugman, 1990). The consequence of this approach to infection control has been the emergence of strains of *S. pneumoniae* with increased resistance to penicillin.

Several pneumococcal infections, such as pneumonia or meningitis, are in many countries still treated with penicillin G. In the treatment of pneumonia, high doses are necessary (Brewin *et al.,* 1974), and mortality is still in the order of 5-20%, particularly in the very young and very old. The mortality rate is independent of resistance to antibiotics, occurring even with appropriate antibiotic therapy. No antibiotic has yet been shown to be superior to penicillin in pneumococcal meningitis but some, such as cefotaxime, may be equally effective.

Of the other β-lactams, only the extended-spectrum cephalosporins, excluding ceftazidime, and the carbapenems are usually equally or more active than penicillin against penicillin-resistant pneumococci. Indeed, it may be the use of ceftazidime that precipitated the emergence of resistance (Linares *et al.,* 1992). Although all pneumococcal strains are susceptible to vancomycin, there is little justification for the use of this agent in pneumococcal pneumonia, especially in light of the recent spread of vanocomycin-resistant enterococci. The macrolides and azithromycin may also be used for pneumococcal pneumonia, but resistance is a problem in certain areas. Most of the older quinolones, like ciprofloxacin, have limited in vitro activity against pneumococci and clinical failures have been described (Gordon & Kauffman, 1990).

However, newer quinolones, like levofloxacin and moxifloxacin, exhibit greater activity against pneumococci.

ii. Treatment of *Haemophilus influenzae* Infections

Until the early 1970s the treatment of choice for *H. influenzae* infections was ampicillin. However, the increasing prevalence of ampicillin resistance in both capsulated and non-capsulated strains has affected the antibiotic therapy for invasive and non-invasive infections (Gunn *et al.*, 1974).

The drug of choice for treatment of invasive infections including meningitis caused by ampicillin-susceptible *H. influenzae* is still ampicillin. For ampicillin-resistant *H. influenzae* strains, many cephalosporins have proved to be highly effective. Nevertheless, the limited susceptibility of this species to most first generation cephalosporins and the poor CSF penetration of these drugs restrict their application to non-invasive infections. The ability of cefaclor to resist the hydrolytic activity of β-lactamases, allow this antibiotic to achieve greater periplasmic concentrations than ampicillin, and sufficient binding to crucial penicillin-binding proteins in *H. influenzae* (Picard & Malouin, 1992). Lower and upper respiratory tract infections caused by *H. influenzae* are treated with ampicillin, co-amoxiclav, cefuroxime and cefpodoxime.

Erythromycin has minimal activity against *H. influenzae*. However, azithromycin and clarithromycin are considerably more active. Tetracycline is active but has unfavorable pharmokinetics and side effects. Newer agents to which *H. influenzae* is highly susceptible are the fluoroquinolones, aztreonam and the carbapenems, but the reliability of CSF penetration has often not been fully established.

1.5.1. The Discovery of β-Lactam Antibiotics

The discovery of the antibacterial action of penicillin and the subsequent demonstration of its therapeutic power led to the production of a large family of antibiotics, whose single common structural feature is the possession of a β-lactam ring. This group of antibiotics includes both penicillins and the cephalosporins, in addition to the newer carbapenems and monobactams. The discovery of 6-aminopenicillanic acid (6-APA) and 7-aminocephalosporanic acid (7-ACA) as the nucleus of penicillins and cephalosporins respectively, and their preparation by enzymatic and chemical methods, followed by chemical modification of the side chains of these antibiotics, have introduced vast numbers of semi-synthetic penicillins and cephalosporins into the chemotherapeutic field. This opened a new era by giving novel compounds effective against various species of bacteria resistant to the early β-lactams.

The widespread use of β-lactam antibiotics stems from their specific inhibitory effect on the biosynthesis of peptidoglycan, a unique structure of bacterial cell walls, and from their low toxicity. Compared to other antimicrobial agents, β-lactam antibiotics can be easily and extensively modified by chemical synthesis. The introduction of specific side chains has resulted in a variety of changes in biological properties, expansion of the antibacterial spectrum, an increase in stability against β-lactamases and improved pharmacokinetic properties.

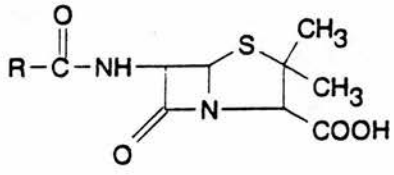
1.5.1.1. Penicillins

In 1929, Alexander Fleming isolated penicillin from a strain of *Penicillium notatum*. By 1941, benzylpenicillin could be produced in sufficient quantity to treat several infected patients. Clinical trials with the agent, conducted by Flory, Chain and colleagues (Chain *et al.*, 1940), were successful, and during World War II, benzylpenicillin was used to treat patients with streptococcal, gonococcal, and treponemal infections. Shortages of the agent continued until the 1940s when production of large amounts of drug became possible by a deep-fermentation

procedure (Fleming, 1946). Since then, many synthetic penicillins have been developed, but resistance to the agents has increased. Despite the emergence of resistance to penicillins and the development of other classes of antimicrobial agents, the penicillins remain one of the most important antimicrobial classes of drugs well into the nineties. In fact penicillin G is still the drug of choice for many types of infections, including syphilis and certain types of endocarditis.

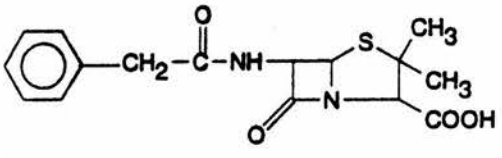
The basic chemical structure of all penicillins consists of a β-lactam ring, a thiazolidine ring, and a side chain (6-APA) (Figure 1.1). The antibacterial activity of the penicillins lies within the β-lactam ring. Any alteration in this ring structure forms penicilloic acid, and the antibacterial activity of the compound is lost. The side chain varies with each penicillin compound and generally determines the spectrum of activity as well as the pharmacokinetic properties of the compound.

Figure 1.1. Structure of Penicillins

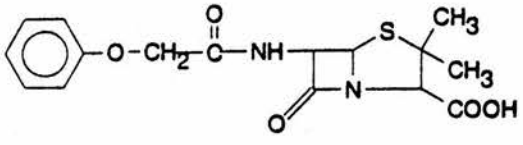


Penicillin Nucleus

(6-aminopenicillanic acid)



Penicillin G



Penicillin V

Classification of Penicillins

The penicillin compounds can be divided into categories based upon their spectrum of activity. Often these early agents are not effective against β-lactamase-producing organisms unless combined with a β-lactamase inhibitor (e.g. clavulanic acid).

i. Natural Penicillins

There are several natural penicillins (penicillin dihydro F, X, and K), of which benzylpenicillin (penicillin G) is the most active and the only natural penicillin used clinically (Figure 1.1).

Penicillin G is produced directly from the fermentation of *Penicillium crysogenum*. Penicillin V is a derivative of penicillin G and, because of similarity in spectrum of activity, is considered a natural penicillin. The natural penicillins have activity against non-β-lactamase-producing gram-positive cocci (Chow & Muder, 1992; Betriu *et al.*, 1994). Activity against gram-negative cocci is limited. However, it is inappropriate to use natural penicillins as empirical treatment for a suspected staphylococcal infections or gonorrhea, due to the increased potential of a resistant organism and subsequent treatment failure.

ii. Penicillinase-Resistant Penicillins

The penicillinase-resistant penicillins are also known as the antistaphylococcal penicillins. Addition of an isoxazolyl side chain to the penicillin compound protects the β-lactam ring from acid hydrolysis by penicillinases produced by *Staphylococcus* spp.

Methicillin, the first agent synthesised in this group, is rarely used currently because of a high incidence of occurrence of interstitial nephritis. Nafcillin, dicloxacillin, oxacillin, flucloxacillin and cloxacillin are the agents commonly used. While less active against streptococci than the natural penicillins, use of the penicillinase-resistant penicillins is

acceptable against these organisms. A notable exception to the gram-positive coverage of this class of penicillins is the enterococci, which are not susceptible to this class of penicillins and activity against gram-negative is nonexistent.

iii. Aminopenicillins

Adding an amino group to the basic penicillin compound led to development of the aminopenicillins. Ampicillin and amoxycillin are the important drugs in this class. The spectrum of activity against gram-positive organisms is similar to that of the natural penicillins. These agents retain activity against streptococci and have slight greater activity against enterococcus than the natural penicillins. The added side chain does not, however, inhibit hydrolysis by staphylococcal penicillinases or gram-negative β-lactamases. The enhanced spectrum of these drugs includes activity against gram-negative bacilli, including *H. influenzae, E. coli, Salmonella* spp. and *Shigella* spp. Presently, however, many strains of these gram-negative organisms are resistant to ampicillin (Schiffer *et al.*, 1974; Spencer *et al.*, 1990; Gross *et al.*, 1981).

iv. Carboxypenicillins

A carboxyl group substitution in place of the amino group yields penicillin compounds that have a greater gram-negative spectrum of action, including activity against *Pseudomonas aeruginosa*, most likely because of increased bacterial penetration through the cell wall. Carbenicillin and ticarcillin are the two drugs in this class.

v. Ureidopenicillins and Piperazine Penicillin

The addition of a ureido group to the penicillin structure produces the compounds azlocillin and mezlocillin, while the addition of ureido group plus a piperazine side chain produces piperacillin. This class of penicillins has a greater gram-negative spectrum of action. The activity against streptococci is slightly less than that of the natural penicillins and ampicillin.

1.5.1.2. Cephalosporins

The first cephalosporin antibiotic was isolated in 1945 from a fungus, *Cephalosporium acremonium*, near a sewer outlet on the Sardinian coast, by Guiseppe Brotzu. In 1953 Abraham and Newton discovered Cephalosporin C (Figure 1.2) while they were studying the antibiotics of a strain of *C. acremonium* (Reviewed by Abraham, 1987). Removal of a side chain from this molecule yielded a nucleus, 7-ACA, from which all subsequent cephalosporins have been derived as semisynthetic compounds (Figure 1.2).

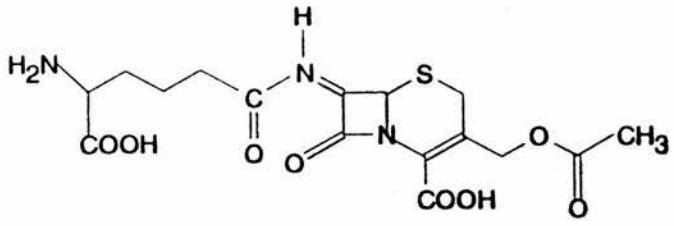
In 1964, cephalothin and cephaloridine were introduced into medicine and were followed by cefazolin. These antibiotics were active against penicillinase-producing *S. aureus* and, unlike methicillin, they also showed significant activity against a number of gram-negative bacilli. In the early 1970s, another family of β-lactam antibiotics was discovered, the cephamycins. Similar to the cephalosporins, these compounds differed in having a methoxy group in the 7-position of the β-lactam ring and being produced naturally by actinomyces rather than fungi.

Thousands of structural modifications have been made to virtually every position on the cephem nucleus. The search for new substitutions at C-3` led to the discovery of quaternary ammonium cephems (cefpirome, cefepime, cefprozan, etc.)

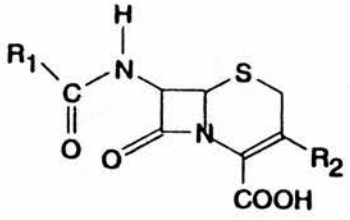
Classification of Cephalosporins

Numerous classifications of cephalosporins have been published based on chemical, biologic, microbiologic, pharmacologic, and immunologic criteria (Belfiglio & Bryskier, 1999; Bryskier & Belfiglio, 1999). No classification has been entirely suitable; nevertheless, the somewhat arbitrary system that is most widely used combines the parental and oral cephalosporins into "generations" on the basis of their antibacterial activity and spectrum of microbiologic activity.

Figure 1.2. Structure of Cephalosporins



Cephalosporin C



Cephalosporin Nucleus

(7-aminocephalosporanic acid)

i. First Generation Cephalosporins

First generation cephalosporins (cefazolin, cephapirin, cephradine, cephalothin, cefaclor, etc.) are very active against gram-positive cocci and have moderate activity against *E. coli, K. pneumoniae, Salmonella* spp., and *Shigella* spp. The antibacterial activity against other *Enterobacteriaceae* is unpredictable and should not be assumed. First generation cephalosporins have poor activity against *H. influenzae*, penicillin-resistant pneumococci, methicillin-resistant staphylococci and enterococci.

ii. Second Generation Cephalosporins

Second Generation Cephalosporins should be considered in two groups: the true cephalosporins (cefuroxime and cefprozil) and the cephamycins (cefoxitin, cefotetan, cefmetazole). The true cephalosporins in this group provide greater activity against staphylococci and nonenterococcal streptococci than the first generation group. In addition, they have significantly improved activity against *H. influenzae* and *M. catarrhalis*. Cephamycins have inferior activity against staphylococci and streptococci.

iii. Third Generation Cephalosporins

These agents (cefotaxime, ceftazidime, ceftizoxime, cefepime, etc.) are commonly viewed as the most potent cephalosporins against facultative gram-negative bacilli. In addition, however, they have superior antimicrobial activity against *S. pneumoniae* (including moderately penicillin-resistant strains), *S. pyogenes, H. influenzae* and *M. catarrhalis*. Most have modest activity against *S. aureus*, with the exception of ceftazidime. This group of cephalosporins, like all of the compounds based on cephem nucleus, lack activity against *Enterococcus* spp., methicillin-resistant staphylococci, and highly penicillin-resistant *S. pneumoniae*. The superior broad activity of these agents against *Enterobacteriaceae* has recently been challenged by β-lactamase and plasmid-mediated resistance, representing a widening threat to the efficacy of third generation cephalosporins.

iv. Fourth Generation Cephalosporins

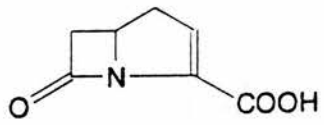
Cefpirome has superior activity against streptococci, *S. aureus, H. influenzae* and the *Enterobacteriaceae* (Wise *et al.,* 1985; Spangler *et al.,* 1994). Cefepime has an exceptionally broad spectrum of antibacterial activity against *S. pneumoniae, S. pyogenes, S. aureus, H. influenzae*, and the *Enterobacteriaceae* (Thornsberry *et al.,* 1993; Yee *et al.,* 1993).

1.5.1.3. Carbapenems

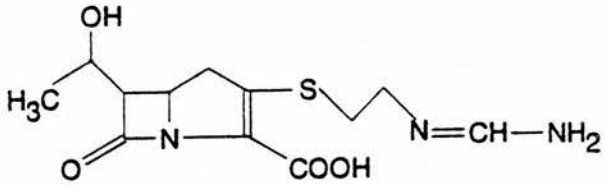
The thienamycins are a group of bicyclic β-lactam compounds that share a common carbapenem nucleus. These naturally occurring antibiotics are produced by the soil organism *Streptomyces cattleya* and were first discovered in the mid-1970s (Albert-Schonberg *et al.,* 1978). There are two main structural differences between carbapenem and penicillin antibiotics: the substitution of carbon atom for sulphur atom at position 1 of the 4:5 fused thiazolidine ring structure, and the presence of an unsaturated bond between C-2 and C-3 (Figure 1.3).

Thienamycin was noted to be stable to β-lactamase hydrolysis and to have a broad spectrum of antimicrobial activity; however it was chemically unstable in concentrated solution. Imipenem (Figure 1.3), a semisynthetic N-formimidoyl derivative of thienamycin, was developed to overcome the chemical instability of the parent compound but was subsequently found to be extensively metabolised by human renal dehydropeptidase I (DHP-I). This enzyme substantially decreased renal excretion of unchanged imipenem and limited the antimicrobial activity of imipenem in urine (Norrby *et al.,* 1983). Cilastatin, a competitive inhibitor of DHP-I with a pharmacokinetic profile similar to that of imipenem, was then developed and combined with imipenem in a 1:1 ratio (Kahan *et al.,* 1983). This combination of imipenem and cilastatin has been available and has proven extremely useful in clinical practice.

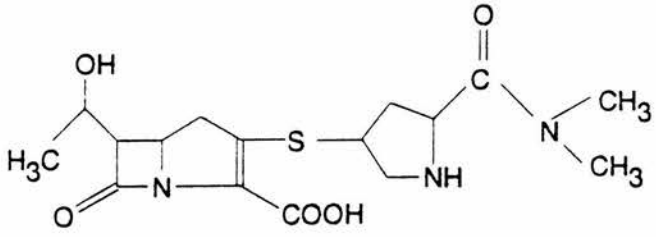
Figure 1.3. Structure of Carbapenems



Nuclear Structure of Carbapenems



Imipenem



Meropenem

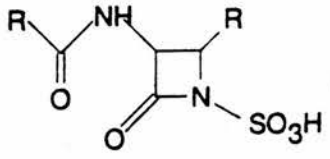
Meropenem (Figure 1.3), also a semisynthetic derivative of thienamycin, is the second carbapenem agent available. Meropenem was developed after chemical modification of thienamycin to provide greater resistant to DHP-I metabolism. The DHP-I stability of meropenem is such that it eliminate the need for coadministration of a dehydropeptidase inhibitor (Moellering *et al.*, 1989).

The carbapenems as a class exhibit the broadest antimicrobial spectrum of any β-lactam antibiotics available to date. They possess excellent activity against both aerobic and anaerobic gram-positive and gram-negative bacteria. Among gram-positive aerobes, the carbapenems are active against *S. pneumoniae* strains with intermediate- or high-level resistance to penicillin; however, these strains are usually four- to eightfold less susceptible than fully penicillin-susceptible strains (Linares *et al.*, 1992). The carbapenems are extremely active against both β-lactamase-positive and -negative strains of *H. influenzae*, including ampicillin-resistant β-lactamase-negative *H. influenzae* (Yeo & Livermore, 1994).

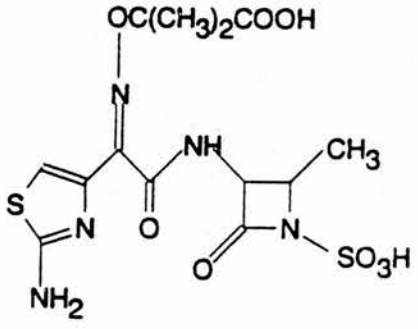
1.5.1.4. Monobactams

The monobactams (monocyclic bacterially derived β-lactams) are a unique class of β-lactam antibiotics whose central core is composed solely of a four-member β-lactam ring and who differ from penicillins, cephalosporins, and carbapenems in that they lack a five- or six-membered side ring (Figure 1.4). They were originally discovered in parallel by two groups of scientist working independently; one for Squibb Pharmaceuticals and the other for Takeda Pharmaceuticals (Sykes *et al.*, 1981; Imada *et al.*, 1981).

Figure 1.4. Structure of Monobactams



Nuclear Structure of Monobactams



Aztreonam

The first monobactams isolated did not exhibit particularly potent antimicrobial activity; however, synthetic side-chain modification resulted in active compounds with increased potency and β-lactamase stability (Sykes & Bonner, 1985). Aztreonam (originally named azthreonam) was among the first synthetic candidates selected for clinical development (Figure 1.4). Since aztreonam is the only representative of its class to be commercially developed, most available information regarding monobactams relates specifically to aztreonam.

Aztreonam has potent activity against most aerobic gram-negative bacteria, including *P. aeruginosa*, but is very limited in its activity against gram-positive bacteria and anaerobes (Jacobus *et al.*, 1982; Barry *et al.*, 1985). Its activity against gram-negative organisms is similar to that of third-generation cephalosporins. Aztreonam is active in vitro against most gram-negative aerobic bacteria because of its resistance to hydrolysis by transferable as well as chromosomal β-lactamases (Livermore & Williams, 1981; Philips *et al.*, 1981). Aztreonam is both a poor inducer and a poor substrate for β-lactamase (Barry *et al.*, 1985).

1.5.1.5. β-Lactamase Inhibitors

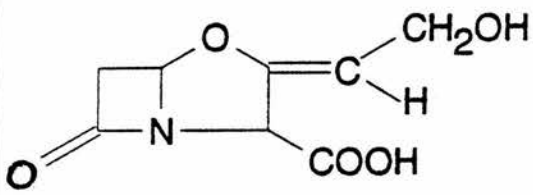
In 1940, penicillin resistance secondary to enzymatic inactivation of penicillin was reported, even though penicillin was not yet widely used. The enzyme, first isolated from *E. coli* by Abraham and Chain, was called penicillinase (Abraham & Chain, 1940). Soon after this discovery, penicillinase was also described in other organisms including *S. aureus*, which by the mid-1940s was widely resistant to penicillin. Following the introduction of penicillin for general use in the 1950s, most isolates of *S. aureus* isolated in hospitals produced penicillinase.

Many unsuccessful attempts to find an inhibitor of this enzyme were made. As early as the 1950s, it was initially that some semisynthetic penicillins, such as oxacillin, could function in vitro as β-lactamase inhibitors, however none proved clinically useful. Olivanic acid and clavulanic acid were discovered as part of a large-scale screening of

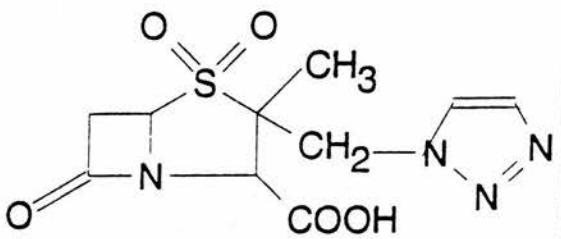
compounds which began in the mid-1960s (Rolinson, 1991). In 1981, clavulanic acid became the first β-lactamase inhibitor introduced into clinical practice, when a formulation that combined it with amoxycillin became available. Since that time, clavulanic acid has been formulated in combination with other broad-spectrum penicillins (e.g., ticarcillin), and other β-lactamase inhibitors, including sulbactam and tazobactam, have been developed (Rolinson, 1991) (Figure 1.5). These antibiotic combination have significantly enhanced spectrum of activity compared with the β-lactam component alone (Livermore, 1993). Resistance due to β-lactamase production occurs in strains of many common organisms such as *S. aureus*, and *H. influenzae*. These agents provide additional therapeutic alternatives for increasingly resistant pathogens.

Most β-lactamase inhibitors developed to date are mechanism-based inhibitors or "suicide" inhibitors of β-lactamases. These compounds, initially recognised as normal substrates by the β-lactamase, eventually form covalent bonds with various amino acid residues within the active site, thus leading to irreversible inactivation of the enzymatic activity. β-Lactamase inhibitors are β-lactam compounds that lack significant antibacterial activity but potentiate the activity of classical β-lactamase-susceptible antibiotics by protecting them from the hydrolytic activity of β-lactamases.

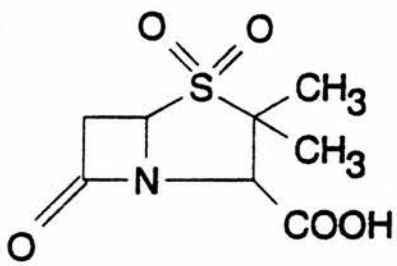
Figure 1.5. Structure of β-Lactamase Inhibitors



Clavulanic Acid



Tazobactam



Sulbactam

1.5.2. The Mode of Action of β-Lactam Antibiotics

The site of action of penicillins was demonstrated by Cooper who observed that radio-labelled penicillin bound to the cell membrane and that consequently the cell died (Cooper, 1956). Tipper and Strominger (1965), later observed that penicillin-treated cells of *S. aureus* possessed more non-cross-linked peptidoglycan in the cell wall than untreated cells. It was therefore concluded that β-lactams inhibit the proteins situated in the cell membrane which catalyse the transpeptidation step of peptidoglycan synthesis so that consequently the cell loses its rigidity and lyses.

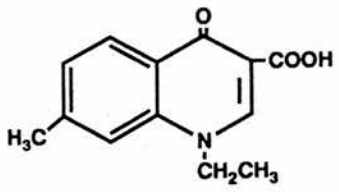
It was realised that there are different groups of proteins involved in the final stages of peptidoglycan synthesis and each preferentially binds to a certain class of β-lactam (Spratt, 1975). This led to the description of the penicillin binding proteins (PBPs) in *E. coli* (Waxman & Strominger, 1983). The usual substrate for PBPs is D-Alanyl-D-Alanine which is very similar in structure to the β-lactam ring. Therefore, the β-lactam can compete with the D-Ala-D-Ala for the active site of the PBP and block the transpeptidation step in cell wall synthesis (Tipper & Strominger, 1965).

1.5.3. The Development of Quinolones As Antimicrobial Agents

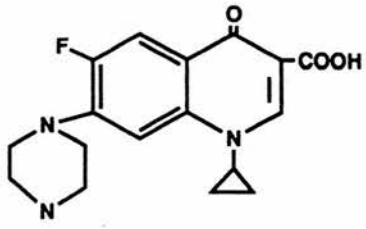
Unlike the β-lactam antibiotics, quinolones are completely synthetic and bear no known resemblance to any compounds found in living organisms. The quinolones have contributed an increasingly important chapter to the evolution of antimicrobials, and their value is still expanding. The story of the quinolones begins in 1962 with the discovery of nalidixic acid, the prototype 4-quinolone antibiotic (Figure 1.6) (Lesher *et al.,* 1962). Nalidixic acid had adequate activity against gram-negative aerobes, but its modest serum and tissue concentrations and its relatively high minimal inhibitory concentrations (MICs) removed the opportunity to treat systemic infections. Therefore, the basic quinolone structure (4-oxo-1,4-dihydro quinolone) was modified to create more potent quinolones, with a broader spectrum and better pharmacokinetic properties. Oxolinic acid and cinoxacin were developed in the 1970s, but these compounds had a narrow spectrum also. The breakthrough came when fluoro-quinolones were developed which have a much greated potency as a result of addition of the fluorine atom at position C7 and a wider spectrum as a result of addition of a piperazine ring at position C6 (e.g., norfloxacin, ofloxacin, ciprofloxacin) (Figure 1.6) (Koga *et al.,* 1980). Ciprofloxacin possessed considerably more potent antibacterial activity than earlier fluoroquinolones.

With extensive chemical modification of the main structure, the resultant group of fluoroquinolones have an improved antibacterial spectrum, favorable pharmacokinetic parameters (including absorption by the oral route and better tissue penetration), lower toxicity profiles, and a reduced tendency to develop bacterial resistance.

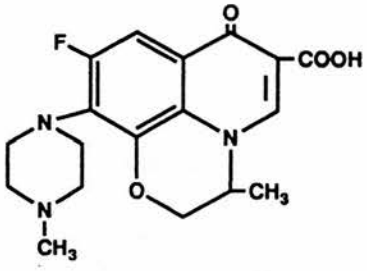
Figure 1.6. Structure of Quinolones



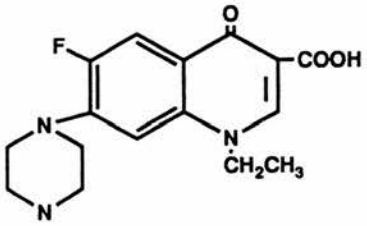
Nalidixic Acid



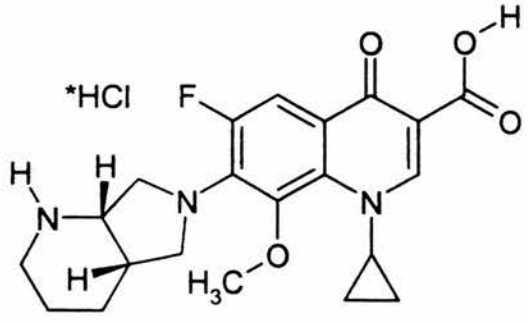
Ciprofloxacin



Ofloxacin



Norfloxacin



Moxifloxacin

Recent structural modifications among the fluoroquinolones include additional fluorine atoms at position 8 and substitutions other than piperazine on position 7. These new compounds enhanced the gram-positive activity of this class at the expense of activity against *Pseudomonas* spp. Fluorine or chlorine atoms at position 8 seem to enhance the phototoxicity of these compounds (Domagala, 1994). A methoxy group in place of fluorine or chlorine at position 8 appears to remove the risk of phototoxicity, as was discovered by the manufacturer of moxifloxacin (BAY 12-8039) (Figure 1.6).

1.5.4. The Mode of Action of Quinolones

Over recent years there has been an enormous explosion of interest in the quinolone drugs in general and the fluoroquinolones in particular (Hooper & Wolfson, 1993 a & b; Kuhlmann *et al.*, 1998).

Gyrase is essential in bacteria as it is involved in a number of cellular processes, including DNA replication and transcription. Therefore, gyrase is an ideal target for antibacterial agents. Evidence that gyrase is the target of the quinolones comes from various types of experiments (Maxwell & Critchlow, 1998). Prior to the discovery of DNA gyrase, early studies had shown that nalidixic acid was an inhibitor of bacterial DNA replication (Pedrini *et al.*, 1972). Following the isolation of gyrase from *E. coli* by Gellert *et al.* (1976), it was shown that its supercoiling activity could be inhibited by oxolinic acid (Gellert *et al.*, 1977). Since that time a large number of quinolone-resistance mutations have been mapped to the gyrase genes, principally to *gyrA* but also *gyrB* (Hooper & Wolfson, 1993 a & b). One example of such a mutation is the Ser83 to Trp mutation of GyrA found in *E. coli* (Yoshida *et al.*, 1988). Enzymes bearing this mutation are resistant to a range of quinolones in vivo and in vitro (Yoshida *et al.*, 1988), and, when complexed with DNA, show a greatly reduced ability to bind the drugs (Willmott & Maxwell, 1993).

DNA gyrase is a member of a family of enzymes called DNA topoisomerases which control the degree of supercoiling in DNA (Wang, 1985). There are two types of topoisomerase, I and II, which are characterised by reactions involving a single- or double-stranded break in DNA, respectively; DNA gyrase is a type II enzyme. Gyrase consists of two subunits, GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes (Wigley, 1995).

Gyrase binds to DNA, and a segment of approximately 130 bp is wrapped around the protein. This wrapped DNA is cleaved in both strands, with a 4-base stagger between the break sites, which results in the formation of DNA-protein covalent bonds between the Gyrase subunits and the 5`-phosphates. Another segment of DNA is passed through this double-stranded break which may then be released. This catalytic supercoiling requires the hydrolysis of ATP. The quinolone drugs interrupt this process at the DNA breakage-reunion step.

Although the evidence that gyrase is the intracellular target of the quinolones is compelling, the existence of a second target, DNA topoisomerase IV (topo IV), has recently been established. Like gyrase, topo IV is a bacteria type II DNA topoisomerase, but unlike gyrase it cannot supercoil DNA (Kato *et al.,* 1990). Topo IV is composed of two subunits, ParC and ParE, which in *E. coli* are encoded by the *parC* and *parE* genes. Evidence for a possible interaction between topo IV and the quinolone drugs has come from various studies (Maxwell & Critchlow, 1998). The function of Topo IV is to decatenate DNA, when replication has occurred, the daughters DNA strands are often interlinked and Topo IV separates them by passing one strand through the other.

In gram-positive bacteria, fluoroquinolones readily pass through the outer areas of the cell membrane and enter the cytoplasm to reach the enzyme. In these organisms, the binding affinity to DNA gyrase appears to be critical to their antibacterial activity. In contrast, gram-negative bacteria have outer cell wall lipopolysaccharide components and porin channels for entry of molecules. There appear to be three mechanisms for

the uptake of fluoroquinolones by gram-negative bacteria. The first is simple diffusion through the outer wall and the cytoplasmic membrane. Fluoroquinolones bind to the cell surface and rapidly diffuse through both components to produce an intracellular pool in the periplasmic space and subsequently within the cell. Fluoroqiunolones can also enter cells via outer membrane protein porins. Finally, it appears that fluoroquinolones chelate magnesium, causing stripping of lipopolysaccharide due to the carbonyl groups at C-3 and C-4. Thus, the hydrophobicity of a specific fluoroquinolone is important in its activity against some gram-negative organisms. There are indications of an active efflux mechanism that removes quinolones from the inside of both gram-positive and gram-negative bacterial cells.

1.6. Development of Antimicrobial Resistance

1.6.1. Mechanisms of Resistance to β-Lactam Antibiotics

Resistance to β-lactam antibiotics can occur by at least three routes. By far the most widespread cause of resistance is the production of a β-lactamase which catalyses the hydrolysis of the antibiotic to a biologically inert product. In gram negative bacteria resistance to β-lactam antibiotics can also result from a decrease in the rate of penetration of the antibiotic through the outer membrane to the PBPs in the cytoplasmic membrane. The third mechanism of resistance to β-lactam antibiotics comes from alterations in the properties of the physiological targets of the antibiotics, the PBPs.

1.6.2. Mechanisms of β-Lactam Resistance in *S. pneumoniae*

1.6.2.1. Penicillin Resistance in *S. pneumoniae*

Classification of penicillin resistance in pneumococci falls into three categories. Fully susceptible isolates have MICs for penicillin ≤0.06 mg/L, MICs for intermediate susceptible isolates are 2- to 20-fold higher than those for fully susceptible ones (i.e.,

0.12-1 mg/L), and isolates with MICs of penicillin >1 mg/L are considered to be resistant (Klugman, 1990).

The absence of β-lactamases in pneumococci and the probable lack of a permeability barrier to β-lactams in all gram positive bacteria infers that resistance to penicillin in pneumococci stems from alterations to their penicillin-binding proteins. Resistance to penicillin in *S. pneumoniae* has been shown to be entirely due to the development of altered forms of the high molecular weight penicillin-binding protein, which have resulted in a reduction in their affinity for the antibiotic (Hakenbeck *et al.*, 1980; Zighelboim & Tomasz, 1980; Spratt, 1989).

1.6.2.2. Penicillin-Binding Proteins in *S. pneumoniae*

Penicillin-binding proteins (PBPs) are cell membrane bound proteins that are present in almost all bacteria. These proteins are associated with cell wall synthesis and are important in cell functions such as division and cell differentiation processes. Major enzymatic activities of PBPs include peptidoglycan transpeptidase and DD-carboxypeptidase. PBPs are targets for β-lactams. Binding of β-lactam antibiotics to PBPs leads to cell lysis, death or growth arrest (Tipper, 1985).

Penicillin-susceptible strains of *S. pneumoniae* contain six PBPs, divided into five high molecular weight PBPs and one low molecular PBPs. PBPs 1a, 1b, 2x, 2a and 2b and the low molecular weight PBP 3, with molecular weights of 92, 89, 82, 81, 74 and 43 kDa respectively (Hakenbeck *et al.*, 1986 a & b; Dowson *et al.*, 1989b & c; Laible *et al.*, 1989).

All six PBPs can occur as low-affinity variants in β-lactam-resistant laboratory mutants (Laible & Hakenbeck, 1987), clinical isolates (Hakenbeck *et al.*, 1980; Zighelboim & Tomasz, 1980; Laible *et al.*, 1991) or transformants obtained with chromosomal DNA of resistant *Streptococcus* spp. (Reichmann *et al.*, 1997; Hakenbeck *et al.*, 1998). The combination of low-affinity PBPs expressed in a

resistant strain varies, perhaps reflecting different selective conditions and indicating a flexibility of the resistance development pathway.

Penicillin resistance in pneumococci has been shown to be due to the sequential alterations of up to three chromosomally encoded high molecular weight PBPs, PBP 2x, 2b and 1a (Dowson *et al.,* 1993). Alterations to these PBPs, particularly to amino acids around the active site serine residue, or around other conserved motifs typically associated with PBPs, substantially reduce their affinity for the antibiotic (Dowson *et al.,* 1993).

1.6.2.3. Penicillin-Binding Proteins of Penicillin-Susceptible & Penicillin-Resistant Pneumococci

The pattern of PBPs on sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels and their affinities for penicillin appear to be similar for different truly-susceptible pneumococci (Hakenbeck *et al.,* 1986 a & b). Isolates with low- or intermediate-level resistance to penicillin appear to retain a normal set of PBPs, but one or more of these have a lower affinity for the antibiotic than the same PBPs from susceptible isolates (Hakenbeck & Tomasz, 1986; Markiewicz & Tomasz, 1989). The PBPs from highly resistant pneumococci have been shown to contain much more complex alterations (Zighelboim & Tomasz, 1980; Markiewicz & Tomasz, 1989). In high level penicillin-resistant pneumococci there have been reductions in the affinity of at least four of the five high molecular weight PBPs (Spratt, 1989; Hakenbeck *et al.,* 1991).

1.6.2.4. The Evolution of Mosaic Penicillin-Binding Protein Genes

The size of the *S. pneumoniae* genome is between 2,240 and 2,700 kbp (Gasc *et al.,* 1991). The fact that the pneumococcal genome chromosome is smaller than that of *E. coli* (Smith *et al.,* 1987) may be related to its many requirements for growth, i.e., 12 amino acids and six vitamins (Sicard, 1964). The genome sizes (1,980 kbp) and growth requirements of *H. influenzae* (Kauc *et al.,* 1989) support this notion. The

reduced size of the pneumococcal chromosome may have resulted from the evolutionary addition of genetic information to a smaller genome or from the elimination of DNA from a more complex organism. This model has been suggested to explain the recent acquisition of new sequences of penicillin-binding proteins (Dowson *et al.*, 1989c).

The exchange of genetic material is restricted by different mechanisms. *Haemophilus*, for example, have evolved uptake mechanisms that interact specifically with sequences that are frequent in the DNA of this species (Danner *et al.*, 1980). *S. pneumoniae*, in contrast, can take up any DNA and the formation of mosaic genes is restricted by the recombination machinery that requires approximately 80% sequence identity between two homologous DNA molecules (Humbert *et al.*, 1995).

In the late 1980s DNA sequence analysis revealed that low affinity PBPs from penicillin-resistant pneumococci had arisen by a novel route, interspecies recombination, presumably mediated by transformation and homologous recombination (Dowson *et al.*, 1989a, b). Unlike penicillin-susceptible isolates of *S. pneumoniae* which possess very uniform PBP genes (<1% sequence variation), penicillin-resistant isolates were found to have very variable PBP genes. They are called "mosaic" genes; since these genes were composed of blocks of nucleotides that were either similar or identical to those from susceptible isolates and blocks that were up to 21% diverged from penicillin-susceptible isolates (Dowson *et al.*, 1989a, b). The genes encoding resistant PBPs 1a, 2x, and 2b consist of mosaics, while the PBP 1b and 2a genes have not yet been sequenced. It was concluded that these mosaic PBP genes were the results of localised recombination events between *S. pneumoniae* and the homologous genes from at least two closely related species (Dowson *et al.*, 1989b). It is now clear that one of the species that has donated PBP 2b genes to pneumococci is *Streptococcus mitis*, which is a commensal member of the oral flora (Dowson *et al.*, 1993). One of the donors of PBP 2x genes is thought to be another member of the oral flora, *Streptococcus oralis* (Sibold *et al.*, 1994). Interestingly, transformation of *S. pneumoniae* from optochin sensitivity; a characteristic trait of the

species, to optochin resistance has also been observed under laboratory conditions using *S. oralis* chromosomal DNA as donor (Fenoll *et al.*, 1994).

Although some mosaic PBP 2b genes are composed of a single block derived from *S. mitis*, others are more complex and are the result of recombination with several donors, possibly each bringing in alterations that further reduce the affinity for penicillin or other β-lactams.

1.6.2.5. Penicillin-Binding Proteins 2B, 2X & 1A

PBPs 2b, 2x and 1a are three of the five high molecular weight PBPs found in *S. pneumoniae* with molecular weights of 74, 82, and 92 kDa, respectively. The *pbp2b, 2x* and *1a* genes encoding PBP 2b, 2x, and 1a have been cloned and sequenced (Dowson *et al.*, 1989a; Laible *et al.*, 1989; Martin *et al.*, 1992) and 2280, 2250, and 3378 -bp open reading frames were identified as encoding PBP 2b, 2x, and 1a, respectively.

PBP 2b is thought to be an important killing target for penicillin (Tuomanen & Sande, 1989) and inactivation of this protein by β-lactam antibiotics has been associated with their ability to cause cell lysis (Hakenbeck *et al.*, 1987). The affinity of PBP 2b for β-lactam antibiotics has been found to be greatly reduced in penicillin-resistant pneumococci (Tomasz *et al.*, 1984).

PBP 2x is thought to be a major target for β-lactams since it is the first PBP altered in β-lactam-resistant laboratory mutants (Laible & Hakenbeck, 1991), as well as penicillin-resistant clinical isolates (Laible *et al.*, 1991). Additionally, PBP 2x has also been shown to be involved in the resistance to third-generation cephalosporins in clinical pneumococcal strains (Muñoz *et al.*, 1992). In conjunction with other high molecular weight PBPs, PBP 2x develops distinct changes in its affinity towards penicillin in pneumococci that have become penicillin-resistant (Hakenbeck *et al.*, 1991). The first three-dimensional crystal structure of PBP 2x has been reported (Pares *et al.*, 1996) and it has been found that the molecule has three domains, the

central domain being a transpeptidase, which is suitable target for antibiotic development.

Comparison of the PBPs from penicillin-susceptible and -resistant pneumococci have shown alterations of PBP 1a to be associated with resistance to penicillin (Markiewicz & Tomasz, 1989; Hakenbeck *et al.*, 1991). This protein presumably plays a role in the development of resistance to penicillin in pneumococci. The *pbp1a* gene was found to map close to the *pbp2x* gene on the chromosome (Gasc *et al.*, 1991). In conjunction with PBP 2x, altered forms of this protein have been shown to confer resistance to third-generation cephalosporins in clinical pneumococcal strains (Muñoz *et al.*, 1992). A low-affinity PBP 1a considerably increases the resistance in a strain with low-affinity PBP 2x and/or PBP 2b (Muñoz *et al.*, 1992; Reichmann *et al.*, 1996).

A low affinity PBP 2b alone confers low levels of resistance to penicillin. However, it does confer another remarkable phenotype, which is reduced lysis upon β-lactam treatment (Grebe & Hakenbeck, 1996). This is reminiscent of the fact that third-generation cephalosporins that do not interact with PBP 2b invoke a tolerant response (Hakenbeck *et al.*, 1987), and a low affinity PBP 2b mimics that effect in regard to other β-lactams. This property, which had been noted in most penicillin-resistant pneumococci, indicates that resistant strains are generally better survivors and can therefore spread more easily than the highly autolytic wild-type strains, and may contribute to the fact that clones of resistant pneumococci are sufficiently stable to be recognisable (Moreillon & Tomasz, 1988; Tuomanen *et al.*, 1988). It also implies that strains with low affinity PBP 2b have a better chance to take up DNA , including genes encoding low affinity variants of other PBPs that are required for higher resistance levels (Hakenbeck, 1998).

1.6.3. Mechanisms of β-Lactam Resistance in *H. influenzae*

1.6.3.1. β-Lactamase-Mediated Resistance

There are nearly 300 β-lactamases found in clinical bacteria and several attempts have been made to classify them (Table 1.1). They are most easily categoriesd by their molecular structure (Ambler class) in which there are four classes; three have serine at the catalytic side and one has a metal ion, usually zinc.

The mode of action of the serine type β-lactamase, such as that found in *H. influenzae* β-lactamase, is shown in (Figure 1.7). Phases of the reaction are: (I) reversible non-covalent binding of the β-lactamase and the β-lactam ring; (II) rupture of the β-lactam ring, which becomes covalently acylated on to the active-site serine; and (III) hydrolysis of the acyl enzyme to reactivate the β-lactamase and liberate the inactivated drug molecule (Livermore, 1998).

The major mechanism of resistance to β-lactam drugs in *H. influenzae* is the production of β-lactamases, and the prevalence of β-lactamase-producing isolates has increased over the last 2 decades. The extent of non-β-lactamase-mediated resistance has always been difficult to assess and it is possible that β-lactamase production may be missed by routine testing (Shanahan *et al.*, 1996).

Three β-lactamases have been described in *H. influenzae*, TEM-1, ROB-1, and most recently, VAT-1. TEM-1 has been found in c. 80% of β-lactamase-positive isolates (Shanahan *et al.*, 1996). Although less numerous than TEM-1, ROB-1 has been found in up to 8% of isolates in some studies (Scriver *et al.*, 1994).

Table 1.1. Molecular and Phenotypic Classification of β-Lactamases

Adopted from Livermore (1998)

Structural Class (Ambler, 1980)	Functional Group (Bush *et al.* 1995)	Richmond-Sykes Class (Richmond & Sykes, 1973)	Substrate preference[a]			
			Penicillin	Oxacillin	cefotaxime	Imipenem
Serine β-lactamases						
A	2a	NL	+++	-	-	-
	2b	II and III	+++	+	-	-
	2be	III and IV[b]	+++	+	++	-
	2br	NL	+++	+	-	-
	2c	II and V	++	+	-	-
	2e	Ic	++	-	++	-
	2f	NL	++	?	+	++
C	1	I, except Ic	++	-	+	-
D	2d	V	++	+++	-	-
Undetermined[c]	4[c]	NL	++	++	V	-
Zinc β-lactamases						
B	3	NL	++	++	++	++

[a] Activity: +++, preferred substrate (highest V_{max}); ++, good substrate; +, hydrolysed; -, stable; V, varies within group; ?, uncertain.

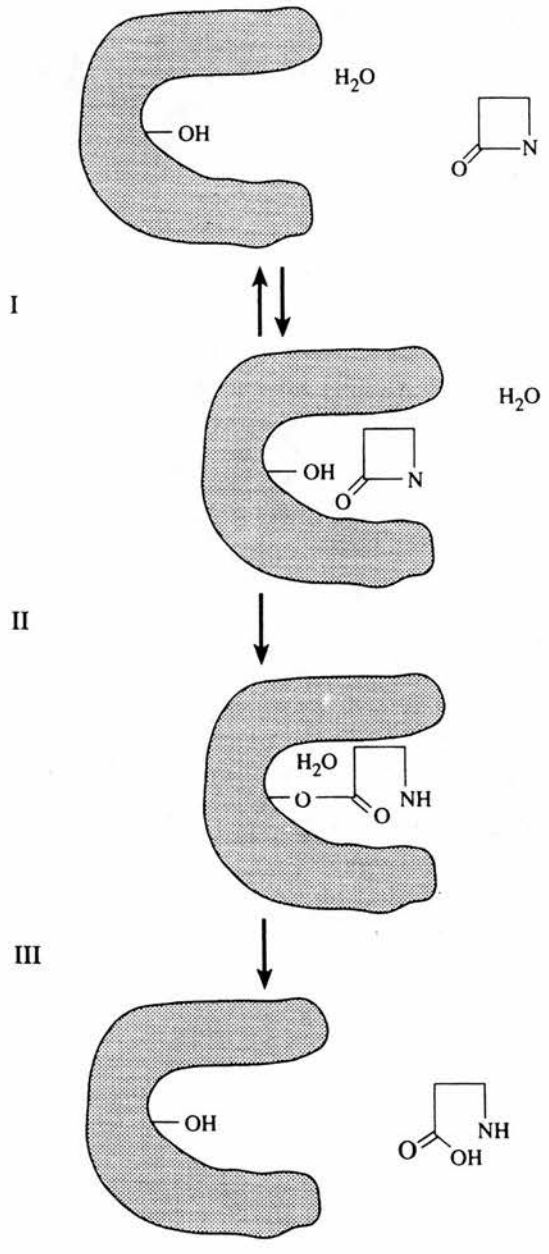
[b] K1 enzyme of *K. oxytoca* was placed in Richmond & Sykes Class IV and Bush Group 2be; however, most Bush 2be enzymes are mutants of TEM and SHV.

[c] None of Bush's Group 4 enzymes has yet been sequenced. They are assumed to be serine types because they lack carbapenemase activity and are not inhibited by EDTA.

NL: not listed.

Figure 1.7. Mechanism of Serine-Based β-Lactamase

Adapted from D.M. Livermore (1998)



i. TEM-1 β-Lactamase

The first reports of clinical resistance to ampicillin in cases of *H. influenzae* meningitis appeared in 1974 (Gunn *et al.*, 1974; Thomas *et al.*, 1974). After that report it was soon shown that the resistance was associated with the presence of a β-lactamase (Williams *et al.*, 1974; Sykes *et al.*, 1975). As judged by its substrate profile and isoelectric focusing (IEF) point (pI 5.4), the β-lactamase present in the resistant isolate is similar to the TEM-1 β-lactamase found in *E. coli* (Sykes *et al.*, 1975). However, DNA sequence analysis of TEM-1 genes (bla_{TEM}) in *H. influenzae* suggests that the bla_{TEM} in this species may differ from its *E. coli* counterpart, and that silent amino-acid substitutions in TEM-1 have occurred (Vali *et al.*, 1995; Vali, 1995). The genetic basis of the TEM-1 β-lactamase-mediated resistance in *H. influenzae* has been well characterised and result indicates that it belongs to Bush group 2b, Ambler class A enzymes (Bush *et al.*, 1995). The β-lactamase is shown to be plasmid-mediated, and restriction enzyme analysis suggests that this enzyme is derived from TEM-1 from Enterobacteria, and that DNA deletion and substitution account for its heterogenicity (Harkess & Morray, 1978).

Some of the plasmids can conjugatively transfer among *H. influenzae* strains, and carry an intact TnA-type transposon as well as other resistance genes. Other plasmids are smaller, are transfer deficient and have undergone deletions of transposon DNA (deGraaff *et al.*, 1976). However, some β-lactamase-producing isolates of *H. influenzae* do not contain plasmid DNA and studies show that the plasmid encoding β-lactamase gene has been integrated into the chromosome (Murphey-Corb *et al.*, 1984).

ii. ROB-1 β-Lactamase

In 1981, a second β-lactamase was reported in *H. influenzae* by Rubin *et al.*, and referred to as ROB-1. Its substrate profile is quite similar to that of TEM-1, but has a much higher pI value of 8.1. It is more strongly inhibited by cloxacillin than is TEM-1.

The ROB-1 gene (bla_{ROB}) is harbored by a 4.4 Kbp plasmid and shows no hybridisation with an intragenic probe for the bla_{TEM} (Medeiros *et al.*, 1986). The ROB-1 β-lactamase is shown to be plasmid-mediated and transferable. A 4.4 Kb ROB-1 plasmid was also found in *Pasteurella haemolytica* (Livrelli *et al.*, 1991), while a 5.4 Kb ROB-1 coding plasmid was isolated from *Haemophilus ducreyi* (MacLean *et al.*, 1992) and also from *Actinobacillus pleuropneumoniae* (Medeiros *et al.*, 1986). All these plasmids are highly related, as measured by DNA hybridisation studies (Medeiros *et al.*, 1986; Livrelli *et al.*, 1991).

The bla_{ROB} gene encoding ROB-1 has been sequenced, and result indicates that it belongs to Bush group 2b, Ambler class A enzymes (Juteau & Levesque, 1990; Bush *et al.*, 1995). The demonstration of similar enzyme in *Pasteurella haemolytica* and *Actinobacillus pleuropneumoniae* suggests that the family *Pasteurellaceae* may be the source of this β-lactamase gene.

iii. VAT-1 β-Lactamase

A completely novel β-lactamase, VAT-1, has also been identified recently in *H. influenzae* (Vali *et al.*, 1994). The significance of this enzyme is not clear at present, but it has been detected in isolates from different geographical centres in Scotland (Shanahan *et al.*, 1996; Vali *et al.*, 1995). The gene encoding VAT-1 has not been sequenced, but biochemical data suggest that it is a Bush class 1, Ambler class C β-lactamase (Bush *et al.*, 1995). VAT-1 exhibits cephalosporinase activity and resistance to the β-lactamase inhibitors clavulanic acid, sulbactam and tazobactam (Vali *et al.*, 1994).

1.6.3.2. Changes of Penicillin-Binding Proteins

In the 1980, ampicillin-resistance in the absence of β-lactamase production was reported in *H. influenzae* (Bell & Plowman, 1980; Markowitz, 1980). The major mechanism of intrinsic ampicillin resistance appears to be due to altered PBPs since reduced affinity of PBPs for ampicillin and other β-lactam agents are observed in such isolates (Parr & Bryan, 1984; Mendelman *et al.,* 1984; Reid *et al.,* 1987). In ampicillin-susceptible *H. influenzae*, a relatively homogeneous pattern of eight PBPs are found (Serfass *et al.,* 1986; Mendelman *et al.,* 1990), whereas intrinsically ampicillin-resistant *H. influenzae* has heterogenous PBP profiles, with five to ten PBPs detected (Serfass *et al.,* 1986; Clairoux *et al.,* 1992). The numbering of the PBPs in *H. influenzae* has become rather confused. Nevertheless, PBPs 3, 4 and 5 appear to be involved in this resistance phenotype. Furthermore, the role of PBP alterations in resistance has definitely been proven by the selection of transformants with similar defects. The genetic basis of this form of resistance is poorly understood but the altered genes are known to be carried on the chromosome. The genes for the altered PBPs have been cloned (Malouin *et al.,* 1987) and results showed that the low penicillin-binding capacity and the β-lactam resistance profiles may be attributed to at least two distinct chromosomal mutations.

1.6.3.3. Outer Membrane Proteins & Permeability

Although it is likely that the alteration of PBPs is the most frequent cause of non-β-lactamase-mediated resistance in *H. influenzae*, decreased outer membrane permeability has also been reported. Burns and Smith (1987) found that some *H. influenzae* resistant to β-lactams lacked a 40 kDa major porin. However, the PBPs of that particular isolate were not investigated. Reid *et al.* (1987) implied that outer membrane profile differences exist between ampicillin-susceptible and -resistant, β-lactamase-negative *H. influenzae* isolates of the same biotype. However, no specific protein change was accounted for this finding.

1.6.4. Mechanisms of Resistance to Quinolones

There are three mechanisms by which microorganisms develop quinolone resistance: (1) a mutational change in the microbial DNA topoisomerase, (2) selected changes in outer membrane proteins, and (3) development of a highly active energy dependent efflux system.

1.6.4.1. Target Sites Modification

Mutations of the *gyrA* gene confer a degree of broad spectrum resistance to all quinolones. Resistance caused by *gyrA* mutation has been reported in the following species: *H. influenzae, S. aureus* and *S. pneumoniae* (Tankovic *et al.,* 1996; Vila *et al.,* 1999). These mutations involve the substitution of amino acids at key enzymatic sites that are involved in the generation of the DNA-gyrase-bacterial complex (Mayer *et al.,* 1995). The effect of these amino acid changes on the secondary and tertiary structures of the GyrA are unknown; however, these alterations affect the affinities of the quinolones for the DNA gyrase-DNA complex. The GyrA protein mutations are closely clustered in a region of about 40 amino acids in the N-terminal domain of the A subunit (Maxwell, 1992). It has been proposed that quinolone binding is cooperative, with at least four molecules binding per site and being associated with each other by hydrophobic interactions between the gyrase via the group at the C7 position of the quinolone (Maxwell, 1992). This quinolone-gyrase-DNA complex forms a barrier to the passage of polymerase.

Mutations can occur in both topoisomerase IV and DNA gyrase in *S. aureus*, and mutations in *gyrA* and *gyrB* can occur in the same strain of *S. aureus*, having an additive effect in raising the resistance level to ciprofloxacin (Gootz & Brighty, 1996). *gyrB* mutations are associated only with low-level resistance (Bryskier, 1993). In addition, quinolone resistance in pneumococci has been found to be caused by mutations in *parC* (Tankovic *et al.,* 1996). Neither plasmid-mediated nor transposon-mediated resistance to the quinolones has been described to date in clinical isolates.

1.6.4.2. Decreased Uptake

The second mechanism involves mutations that affect the permeability of the cells to the quinolone, therapy resulting in reduced uptake into the microbial cell. This may be the result of diminished production of the outer membrane proteins, particularly OmpF, of gram-negative microorganisms (Piddock *et al.*, 1991) and is of particular concern because the decreased permeability leads to cross-resistance with structurally unrelated antimicrobial agents. In contrast, decreased uptake has not been demonstrated to be a mechanism of resistance in gram-positive bacteria.

Hydrophobic quinolones (e.g., nalidixic acid) pass directly through the lipid bilayer of the cell membrane whereas hydrophilic quinolones (e.g., ciprofloxacin) pass through water-filled protein pores called porins. In *E. coli*, the inactivation of the *ompF* gene and mutations in the regulatory genes controlling expression of the major porin *ompF* gene can cause resistance to quinolone as well as other non-related groups of antibacterials (Cohen *et al.*, 1989). Reduction of the OmpG production has been reported in *P. aeruginosa* (Chamberland *et al.*, 1989).

1.6.4.3. Increased Efflux

This mechanism prevents effective intracellular concentrations of the quinolones from being attained and involves an active efflux system at the inner membrane. Efflux occurs through the cytoplasmic membrane into the periplasmic space probably via an energy-dependent active transport system (Bryskier, 1993). Although not identified for all quinolones, this mechanism occurs in both gram positive and gram negative microorganisms. Efflux seems to act in conjunction with a second mechanism, possibly one causing reduced diffusion across the outer membrane through porin channels (Mayer *et al.*, 1995). In *S. aureus*, the *norA* gene encodes for a multiple antibiotic efflux pump (Neyfakh *et al.*, 1993). In addition, data from a recent study by Brenwald *et al.*, indicates that a multidrug efflux mechanism may also occur in mutant strains of *S. pneumoniae* (Brenwald *et al.*, 1997).

1.7. Incidence & Spread of Antimicrobial Resistance

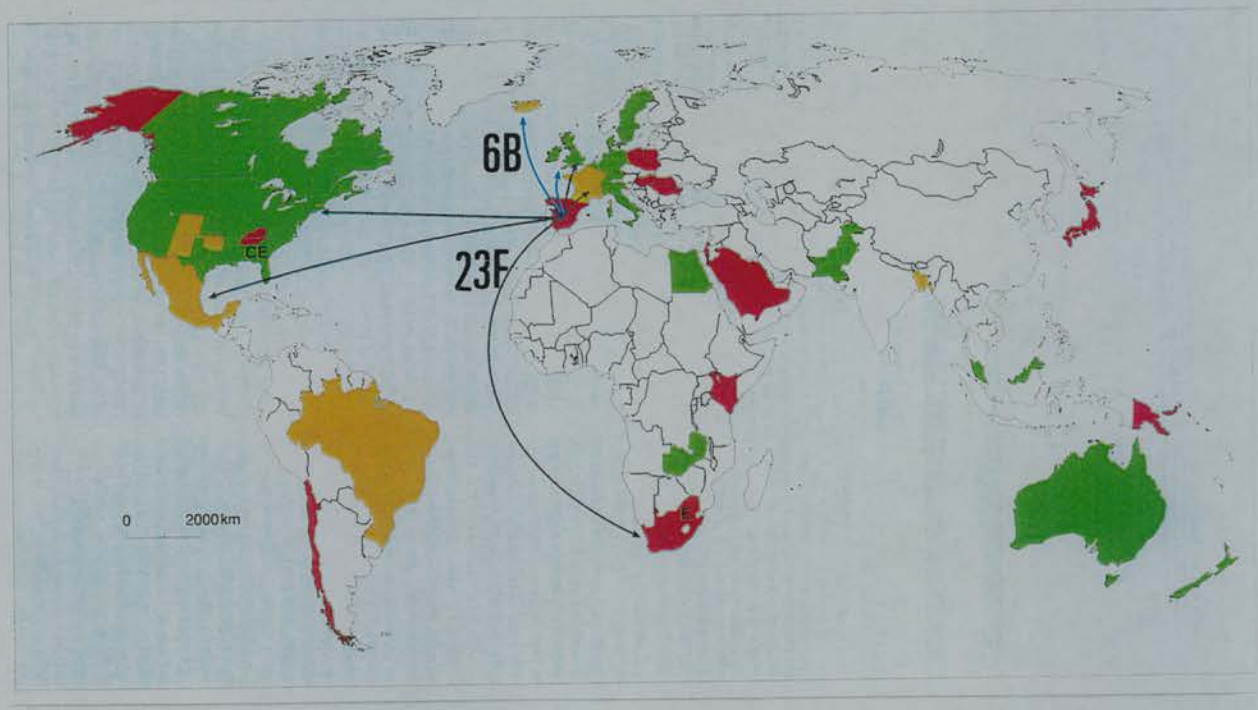
1.7.1. Incidence of Resistance in *S. pneumoniae*

The origins of penicillin-resistant pneumococci remain unclear, and they have probably emerged on many occasions at diverse geographical locations. They were first recorded in the late 1960s in Australia, Papua New Guinea and the USA (Hansman & Bullen, 1967; Hansman *et al.*, 1971; Naraqi *et al.*, 1974). Since then, penicillin-resistant isolates (with MICs ≥0.1 mg/L) have been reported worldwide (Appelbaum, 1987) with particular high incidences among invasive isolates from Hungary, South Africa and Spain (Klugman, 1990) (Figure 1.8). There has also been a marked increase in the levels of resistance to penicillin. In Spain more than 15% and in Hungry more than 48% of the total number of penicillin-resistant isolates are highly resistant (MICs >1 mg/L) (Fenoll *et al.*, 1991; Marton *et al.*, 1991), and isolates with MICs of 16 mg/L have been found recently in central Europe.

Until recently the UK was considered to have low prevalence of penicillin-resistant pneumococci. As recently as 1990, 100% of 7255 strains of pneumococci from 61 centres across the UK were found to be penicillin susceptible (Spencer *et al.*, 1990). However, there have now been several reports of significant and rising levels of resistance nationwide (Goldsmith *et al.*, 1997). In Northern Ireland, 3171 strains of *S. pneumoniae* were examined, during which time the annual rate of penicillin resistance was found to increase from <1% to 10.6% (Goldsmith *et al.*, 1997). Similar rates of penicillin resistance have now been reported from several geographically disparate regions in the UK (Goldsmith *et al.*, 1997). Clearly both penicillin-resistant and multidrug-resistant pneumococci are increasing in prevalence in the UK, and this increase is likely to continue.

Figure 1.8. The Clonal Spread and World-Wide Prevalence of Penicillin-Resistant *S. pneumoniae*

Adopted from Dowson *et al.* (1994)



Prevalence of penicillin resistance: **<10%** **10-20%** **>20%**

The spread of the multidrug-resistant serotype 23F clone from Spain to the USA, Mexico, France, Portugal, South Africa, Poland and the UK is illustrated by the dark-blue arrows. Countries in which serotype 23F variants are found that show high resistance to cephalosporins (C) and erythromycin (E) are shown. The light-blue arrows show the spread of the multidrug-resistant serotype 6B clone from Spain to the UK and Iceland.

A low incidence of resistance is found in several other countries of Northern Europe and elsewhere. Continuing surveillance is essential in those countries in which resistant pneumococci are rare, since these strains can rapidly become entrenched within a community. A good illustration is provided by studies from Iceland where penicillin-resistant pneumococci were unknown until 1989 when a multiresistant serotype 6B pneumococcus appears to have been imported from Spain: within 4 years, 20% of pneumococcal infections in Iceland were due to this multiresistant strain (Soares *et al.,* 1993). Similarly, in Hong Kong the incidence of penicillin resistance in pneumococci from sputum has increased from 6.6% in 1993 to 55.8% in 1995 (Lyon *et al.,* 1996). Another example has been observed in the USA, where low incidence of penicillin resistance appeared in 1980s but recent surveys have shown an overall incidence of penicillin resistance among invasive isolates to have increased to 25%, with much higher proportions of resistant isolates in some areas (e.g. 70% in Tennessee). Most of the rise in resistance also appears to be due to the establishment of imported Spanish multiresistant pneumococci.

Multiple resistance, defined as resistance to at least three different classes of antibiotic, is frequently encountered in penicillin-resistant isolates (Appelbaum, 1987). The emergence of multiply-resistant pneumococci was first observed from South Africa (Jacobs *et al.,* 1978). A strain from a paediatric patient with pneumonia was resistant to penicillin, erythromycin, tetracycline, chloramphenicol, clindamycin and TMP-SMZ. A subsequent study in South Africa showed the prevalence of multiple-resistant pneumococci to be as high as 56.5% in some areas (Koornhof *et al.,* 1978). In Spain, resistance to chloramphenicol, tetracycline and TMP-SMZ was observed in 83% of the penicillin-resistant isolates causing pneumonia, and also in up to 33% of the penicillin-susceptible strains isolated from control patients (Pallares *et al.,* 1987).

Other antimicrobial agents, such as erythromycin, TMP-SMZ, tetracycline, and chloramphenicol may be used to treat pneumococcal infections, but resistance to these agents usually increases in parallel with penicillin resistance rates, and multiresistant strains have been problematic in some communities. There is cross resistance among

the macrolides, and erythromycin-resistant strains are also resistant to clarithromycin and azithromycin (Moreno *et al.,* 1995). Vancomycin resistance has not been described among pneumococci. Early-generation fluoroquinolones are not particularly active against pneumococci, but newer quinolones such as levofloxacin, and trovafloxacin are highly active against both penicillin-susceptible and -resistant pneumococci (Visalli *et al.,* 1996). In contrast to many other antimicrobial agents, there is usually no association between penicillin resistance and susceptibility to vancomycin or the quinolones.

1.7.2. Spread of Resistance in *S. pneumoniae*

Resistance to penicillin in *S. pneumoniae* has been shown to be entirely due to the development of altered forms of the high molecular weight PBPs, which have resulted in a reduction in their affinity for the antibiotic (see section 1.6.2).

Penicillin-binding-protein-mediated resistance to penicillin can spread either by the dissemination of resistant organisms (clonal spread) or by the dissemination of mosaic genes (horizontal spread) (Dowson *et al.,* 1994; Crook & Spratt, 1998). Thorough analysis of these organisms requires the use of techniques that index both their overall genetic relatedness (e.g. multilocus enzyme electrophoresis) and the relatedness of their altered penicillin-binding protein genes (e.g. gene fingerprinting).

1.7.2.1. Clonal Spread of Drug-Resistant Pneumococci

The Major Spanish Clones of Antibiotic-Resistant Pneumococci

The best characterised, and most globally distributed, penicillin-resistant strains are those that originated within Spain. Two multiresistant strains become prevalent in Spain during the 1980s.

One of these, the multiresistant serotype 23F clone (resistant to penicillin, tetracycline, chloramphenicol and cotrimoxazole) probably arose in Spain at least 15 years ago, and has been identified subsequently in at least six other countries across three continents (Coffey *et al.*, 1991; Muñoz *et al.*, 1991; Sibold *et al.*, 1992) (Figure 1.8). As members of this resistant clone have spread, they have been exposed to new selective pressures applied by regional variations in usage of the different classes of antibiotics. This has led to variants of the 23F clone that are resistant to erythromycin in South Africa (Klugman *et al.*, 1994) and in the USA (McDougal *et al.*, 1992) also, in the USA, to variants that have increased resistance to extended-spectrum cephalosporins (McDougal *et al.*, 1995) (Figure 1.8).

The second major multiresistant Spanish serotype 6B clone, has a similar resistance profile and appears to have spread slightly less than the 23F clone. It has, however, been found in the UK and several European countries, the Far East and the USA, and has been particularly successful in Iceland (Soares *et al.*, 1993) (Figure 1.8).

1.7.2.2. Horizontal Gene Transfer in Pneumococci

Analysis of the overall relatedness of isolates and that of their mosaic *pbp* genes has revealed the huge diversity of penicillin-resistant pneumococci and the independent origins of the resistant isolates from Spain and South Africa. The diversity of penicillin-resistant pneumococci is evident both from an analysis of their mosaic *pbp* genes and from the genetic backgrounds in which these genes are found (Dowson *et al.*, 1997; Crook & Spratt, 1998). Some of the diversity is probably due to the

multiple origins of mosaic *pbp* genes, but a major part within countries may be due to the distribution of mosaic *pbp* genes between pneumococcal lineages by horizontal gene transfer. Horizontal gene transfer has been invoked to explain the presence of identical mosaic *pbp* genes in penicillin-resistant pneumococci from genetically distinct lineage.

A third Spanish clone of serotype 9V, that is resistant only to penicillin and cotrimoxazole, has also spread globally and possesses the identical penicillin-binding protein genes (*pbp1a, 2b* and *2x*) as the 23F clone, although it has a very different overall genotype (Coffey *et al.*, 1991). The 9V clone has probably emerged by the horizontal spread of the altered *pbp* genes from the 23F clone into a penicillin-susceptible serotype 9V pneumococcus (Coffey *et al.*, 1991).

1.7.2.3. Serotype Changes Among Penicillin-Resistant Pneumococci

Several groups have provided evidence that members of a single penicillin-resistant clone may express distinct capsular polysaccharides. The existence of penicillin-resistant clinical isolates that are identical in overall genotype, in their antimicrobial resistance profile, and which posses identical altered forms of the *pbp1a, 2b* and *2x* genes, except capsular type has led to the suggestion that horizontal transfer of genes involved in capsular biosynthesis may be important in generating penicillin-resistant clonal variants of differing serotypes. For example, serotype 19F isolates from Spain and the USA are indistinguishable from members of the multiresistant serotype 23F clone, except for their capsular type (Coffey *et al.*, 1991; McDougal *et al.*, 1992). Another example of putative serotype changes among penicillin-resistant pneumococci was found in isolates from Kenya (Kell *et al.*, 1993).

1.7.2.4. Global Distribution of Resistant Clones

Several general conclusions can be drawn from the molecular epidemiological studies. Firstly, even within a single country, there is a considerable diversity of penicillin-resistant strains. Many penicillin-resistant strains appear to be unique isolates, but among this diversity there are some strains that are recovered repeatedly, and which in some cases are spreading globally (Coffey *et al.,* 1995; Tomasz, 1997). Secondly, the majority of resistant isolates belong to relatively few serotypes, which are those commonly associated with carriage and disease in children (e.g. 6B, 14, 19A, 19F, 23F) (Klugman, 1990). Thirdly, the resistant strains recovered in different countries are often unrelated. For example, the multiresistant strains recovered within Spain, Hungry and South Africa are completely distinct, and almost certainly emerged separately in each of these countries. This distinction is now becoming slightly blurred as successful resistant clones are spreading globally, and also does not apply to neighboring countries which often share the same resistant pneumococci (Klugman, 1990).

1.7.3. Incidence of Resistance in *H. influenzae*

1.7.3.1. Overall Incidence

Resistance to ampicillin was first documented in Europe in 1972 (Mathies, 1972). Since then, considerable information has been gathered with regard to its prevalence throughout the world.

Among the mechanisms mentioned (see section 1.6.3.1) production of TEM-1 β-lactamase is by far the most common cause of ampicillin resistance encountered in *H. influenzae* globally, especially among *H. influenzae* type b (Powell *et al.,* 1987; Jorgensen *et al.,* 1990; Collignon *et al.,* 1992). Among the β-lactamase producers, 7% produced ROB-1 enzyme in a recent survey in Canada (Scriver *et al.,* 1994), and 8% in a survey in the USA (Daum *et al.,* 1988) but only about 0.6% in a survey in France (Joly *et al.,* 1987). Intrinsically ampicillin-resistant *H. influenzae* accounts for

<5% of isolates and are more common among non-capsulated organisms (Howard & Williams, 1988 & 1989; Powell *et al.*, 1987).

Since the last decade, the prevalence of β-lactamase production in the UK has increased significantly from 6.2% in 1986 (Powell *et al.*, 1987) to 8.6% in 1991 (Powell *et al.*, 1992). Similarly, the incidence in the USA has also increased in the past ten years (Jorgensen *et al.*, 1990; Barry *et al.*, 1994). In Europe, β-lactamase-producing isolates appear to be confined to certain areas. Machka *et al.*, (1988) published data on the susceptibility of *H. influenzae* isolated from nine countries in 1986, which showed that Spain had the highest incidence of β-lactamase-producing isolates (35%). In other European countries, the incidence of β-lactamase isolates was 27% in Belgium, 11% in France, 6% in Austria, 2% in Germany and 6% in Switzerland. In addition, the overall rate of β-lactamase production was 16% in Australia (Collignon *et al.*, 1992) and 28.4% in Canada (Scriver *et al.*, 1994).

During the 1980s, as result of excellent in vitro activity and CSF penetration, third-generation cephalosporins became the preferred treatment for invasive *H. influenzae* disease (Campos & Garcia, 1987). First-generation cephalosporins (e.g., cefaclor and cephalexin) have limited activity against *H. influenzae* (Powell *et al.*, 1991). Cefaclor resistance has a prevalence of 1.4 to 5.5% in the USA and 2% in Europe. Second- and third-generation oral cephalosporins, cefuroxime axetil, cefixime and cfepodoxime proxtil have MICs below 0.5 mg/L. Resistance is generally less than 2% for cefuroxime and cefamandole, and resistance to newer cephalosporins has not been documented (Rockowitz & Tunkel, 1995). Cefotaxime, ceftazidime and ceftriaxone all have excellent CSF penetration and are extraordinarily active against *H. influenzae* (Jacobs & Kearns, 1989). β-Lactamase producers are susceptible to β-lactam antibiotic/β-lactamase inhibitor combinations and many newer cephalosporins. The β-lactamase inhibitors clavulanate, sulbactam, and tazobactam are active against *H. influenzae* harboring TEM-1 and reduce the MIC by 8-to 64-fold.

In the UK, erythromycin has little activity against *H. influenzae*. Resistance to chloramphenicol and tetracycline has been detected, but is currently <5% for each (Brown *et al.*, 1996). Current resistance level to trimethoprim is 9.6% (Brown *et al.*, 1996). Although there are a number of isolated reports of the emergence of quinolone resistance in *H. influenzae* (Gould *et al.*, 1994), this does not appear to be a widespread phenomenon and resistance to ciprofloxacin and other quinolones remains low.

Problems with the therapy of infection due to bacterial isolates with "intermediate" susceptibility has been shown to occur in *S. pneumoniae* (Klugman, 1990). If this is found to be of clinical importance with *H. influenzae*, there will be major problems in the therapy of infections caused by such organisms. Moreover, this type of reduced susceptibility often extended not only to co-amoxiclav, but also to other β-lactams.

1.7.3.2. Incidence in Capsulated and Non-Capsulated *H. influenzae*

H. influenzae type b isolates were statistically much more likely to be β-lactamase-positive than the non-capsulated isolates, this is in agreement with the results of the UK surveys of 1986 (Powell *et al.*, 1987) and 1991 (Powell *et al.*, 1992). These patterns have also been described elsewhere (Doern *et al.*, 1988; Collignon *et al.*, 1992). In concordance with the findings of these authors, type b isolates were statistically more likely to be isolated from body fluids than from other sources and were more often isolated from patients under the age of five than from other age groups (Nazareth *et al.*, 1992).

1.7.3.3. Incidence in Non-β-Lactamase-Mediated Resistance

The incidence of non-β-lactamase-mediated-resistance to ampicillin (MIC ≥1 mg/L) in *H. influenzae* isolates has continued to rise in the last few years in the UK; from 4% in 1986 (Powell *et al.,* 1987) to 5.8% in 1991 (Powell *et al.,* 1992). Among other international studies, only Australia has previously reported a high resistance rate, where 7% of isolates were resistant to ampicillin in the absence of β-lactamase production (Bell & Plowmann, 1980). Such strains remain rare, accounting for fewer than 1% of all isolates elsewhere (Barry *et al.,* 1994; Scriver *et al.,* 1994). However, many authors only included those isolates for which the ampicillin MIC exceeded 1 mg/L as resistant, and isolates for which the MIC was 1 mg/L as intermediate. If an ampicillin breakpoint MIC of ≥2 mg/L were applied, the present rate of non-β-lactamase-mediated isolates with reduced susceptibility to ampicillin would fall to 2.7%, which is more closely in agreement with data from elsewhere.

1.8. Application of Techniques to Determine the Incidence, Mechanism and Spread of Resistance

To be a useful epidemiologic tool, a typing system must give an unambiguous result for each isolate (typeability), give the same result each time the same isolate is tested (reproducibility), and differentiate among epidemiologically unrelated strains (discriminatory power). The earliest typing methods used for epidemiologic purposes were phenotypic methods. Phenotypic methods detect characteristics expressed by microorganisms in response to antibiotics or other inhibitors, as a product of one of their enzymes, or as a protein on their cell surface. Phenotypic methods are not as stable as DNA-based methods. In addition, phenotypic methods tend to lump isolates together in large groups, and thus, do not discriminate between strains as well as DNA-based methods. Genotypic typing methods that evaluate differences at the DNA level are used more commonly because they are more discriminatory than phenotypic methods. In addition, all isolates should be typeable by tests evaluating the chromosomal DNA and the results are usually reproducible, which cannot be said of phenotypic methods.

1.8.1. β-Lactam Resistance Mediated by Changes in PBPs

PBP-mediated resistance occurs when the resident PBPs are altered to forms where they have a substantially reduced affinity for penicillin or other β-lactams, as in the evolution of penicillin-resistant strains of *S. pneumoniae* and *H. influenzae*.

1.8.1.1. Detection of Resistance to β-Lactams

The upward trend of β-lactam resistance in *S. pneumoniae* emphasises the need for a reliable and convenient method for its detection. For the routine testing of penicillin susceptibility of pneumococci, the use of 1 μg oxacillin discs is thought to be a reliable method, and its currently recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1990) and the British Society for Antimicrobial Chemotherapy (BSAC, 1998). This method does not give a truly quantitative susceptibility determination, and it is difficult to distinguish between the

transitions from low-level penicillin resistance, to intermediate-level resistance, and to high-level resistance. Such distinctions are clinically important for treatment decisions for pneumococcal infections (Paris *et al.,* 1995). The E-test is thought to be one of the most accurate of the commercial tests that are available (Tenover *et al.,* 1996), and a reliable alternative to agar or broth dilution methods.

Several DNA amplification-based techniques have been described that use *pbp* gene PCRs as targets (Ubukata *et al.,* 1996; Jalal *et al.,* 1997). Alternatively, a restriction fragment length polymorphism (RFLP) strategy has also been investigated and has been shown to be valuable in differentiating between penicillin-susceptible and -resistant pneumococcal strains and can be used to complement PCR diagnosis (O'Neill *et al.,* 1999).

1.8.1.2. Detection of PBPs & Their Affinity for β-Lactams

PBPs profiles can be detected by incubating whole cells or membrane preparations either directly with radiolabelled penicillin, or in competition with unlabelled compounds, then resolving them in SDS-PAGE and visualising them by fluorography (Spratt, 1977). This technique does not look directly at the PBP genes but the proteins that they encode, revealing any variation in the number and molecular size on SDS-PAGE. This technique has different limitations, is time consuming and requires usage of radiolabelled compounds. In addition, several earlier studies were unable to resolve each of the PBPs and, hence, this makes the results difficult to interpret. Reproducibility between different results requires a strict adherence to protocol as even a change in the manufacturer of the SDS used for PAGE can alter the final fluorogram. Apart from this, altered low-affinity PBPs can migrate either fast or slow to give an apparently altered molecular weight when compared with their high affinity forms (Markiewicz & Tomasz, 1989).

1.8.1.3. Epidemiologic Investigation

In species where recombination is common, as in *S. pneumoniae*, one has to be careful in attributing inappropriate epidemiologic weight to phenotypic methods such as serotyping or antibiotic resistance patterns when trying to determine whether resistance has spread clonally or by horizontal gene transfer. This distinction can be achieved using a combination of methods such as multilocus enzyme electrophoresis (MLEE), ribotyping, pulsed-field gel electrophoresis (PFGE) and gene fingerprinting or partial gene sequencing that are able to index either the overall genetic relatedness between isolates or the relatedness of their resistance determinants. Resistant pneumococci, that are not closely related, can possess identical altered *pbp* genes and isolates that are apparently genetically identical can possess different *pbp* genes (Dowson & Coffey, 1998).

1.8.2. β-Lactam Resistance Mediated by Production of β-Lactamases

β-Lactamases confer resistance to β-lactam antibiotics, which are the most widely used family of antibiotics. It is, therefore, essential that one can identify the production of β-lactamases by clinical isolates and have effective ways of distinguishing the different enzymes. This is necessary for epidemiologic surveys, predicting future resistance trends, and to ensure that patients receive the appropriate β-lactam or alternative therapy.

1.8.2.1. Biochemical Application for Characterisation of β-Lactamases

For the past 20 years thin-layer polyacrylamide isoelectric focusing (IEF) has played a major role in the identification and characterisation of β-lactamases (Matthew *et al.*, 1975). IEF is able to distinguish enzymes that focus only 0.05 pI apart (Payne *et al.*, 1989), but the exponential increase in the numbers of β-lactamases discovered over the last 10 years (>200 β-lactamases) has meant that this method no longer provides

sufficient resolution to distinguish the majority of β-lactamases. Today the pI of a β-lactamase is still an essential determinant that must be used in combination with a variety of other data. Moreover, the IEF of β-lactamases is now entering a new era as this technique can be adapted to provide important biochemical information on β-lactamases other than simply their pI values (Payne & Farmer, 1998).

1.8.2.2. Molecular Approaches for the Detection & Identification of β-Lactamases

Molecular techniques have become very effective for identifying different β-lactamases (Payne & Thomson, 1998). However, in some cases this approach must still be combined with kinetic and IEF data to identify a β-lactamase.

The first indication of the presence of β-lactamases is usually the observation of increased levels of resistance in clinical isolates as detected by routine susceptibility testing. In some species, like *H. influenzae*, the presence of a β-lactamase in itself is enough information to make a judgment on treatment options. IEF then can be used to characterise the β-lactamase produced. However, the whole approach is time consuming, in terms of enzyme extract preparation and running electrophoresis, and the need for large amount of cells for enzyme extraction.

PCR has been extensively used in the detection and analysis of resistance genes in bacterial isolates (Jalal *et al.*, 1997) and in the detection of specific microorganism directly in clinical and environmental specimens (Shirai *et al.*, 1991). However, it is only relatively recently that the possibility of employing PCR in the direct detection of resistance genes in clinical specimens has been investigated. PCR has been used to detect ampicillin resistance genes in CSF samples containing *H. influenzae* (Tenover *et al.*, 1994). PCR primers were used that were specific for the bla_{TEM} and bla_{ROB} genes. Correlation was obtained between the result of MIC testing, β-lactamase production as determined by nitrocephin and PCR testing. The primary advantage of

PCR is that it detects and amplifies very small amounts of DNA in a short amount of time. However, any contaminating DNA can produce false-positive results.

1.8.3. Molecular Characterisation of Quinolone Resistance

Unlike the β-lactams, it is not possible to examine the resistance pattern to a cohort of quinolones and predict the resistance mechanism. Resistance to one of the group is almost always accompanied by a proportional increase in resistance to the others, which means that distinction cannot be made by observing differential increases in resistance.

Recently, PCR has been used to amplify the quinolone resistance-determining region (QRDR) of the *gyrA* gene and, more latterly, the *parC* gene. This takes oligonucleotides that match conserved sequence and part of the gene, of known and measurable size, is amplified. Subsequently, it is possible to separate the DNA strands and directly sequence them. This is not feasible in a diagnostic laboratory as, although fairly rapid nowadays, the use of a radiolabel, for sequencing, and the time taken for analysis makes it impractical. If a suitable restriction site is found, then it is possible to distinguish alterations in a specific codon. This is much more practicable, and it is quite possible to prepare amplified DNA and examine the RFLP within one day.

1.9. Prevention-2

Antibiotic Usage Control & Surveillance

Excess usage of antibiotics is seen as a major factor in the emergence of resistant bacteria (Courcol *et al.*, 1989). The hope is that control of antibiotic usage will contain the rise in antibiotic resistance or even reverse the trend (Cohen, 1994). Though plausible, this still needs to be proven as a reliable public health approach to the control of resistance without the disadvantage of under treating infectious disorders. The recognition and impact of resistant bacteria on the burden of bacterial disease can only be determined by rigorous population based and active surveillance (Hughes & Tenover, 1997). Such data would also potentially provide information useful in devising and modifying strategies of antibiotic usage control or even recognising alternative approaches to limiting the spread and impact of resistant bacteria.

1.10. Aims of this Thesis

Streptococcus pneumoniae

1. What is the level of penicillin resistance in *Streptococcus pneumoniae* isolates and how does this correlate with resistance to other antibiotics?

2. What is the relationship between classical serotyping of *Streptococcus pneumoniae* and more exacting molecular typing procedure?

3. In the emergence of penicillin resistance, is this caused by the clonal spread of a few "epidemic" strain or results from spontaneous emergence in individual patients?

4. What effect does resistance in *Streptococcus pneumoniae* have on the ability to treat with fluoroquinolones?

Haemophilus influenzae

1. What is the level of amoxycillin resistance in *Haemophilus influenzae* isolates?

2. What are the mechanisms of penicillin resistance in amoxycillin-resistant isolates of *Haemophilus influenzae*?

3. Is the spread of *Haemophilus influenzae* clonal?

CHAPTER TWO

Materials & Reagents

2.1. Bacterial Strains

The bacterial strains used in this study are shown in Table 2.1. The purity and integrity of the *S. pneumoniae* and *H. influenzae* strains were initially verified by colony morphology and then by standard microbiological tests.

The clinical isolates of *S. pneumoniae* and *H. influenzae* were collected from the following centres throughout the UK:

	S. pneumoniae (n= 70)	*H. influenzae* (n= 231)
Royal Infirmary of Edinburgh (RIE)	10	20
Bolton Royal Infirmary (BRI)	3	11
North Middlesex Hospital (NMH)	11	21
Dulwich Hospital (D)	7	14
Whiston Hospital (WH)	6	18
Freeman Laboratories (FL)	5	9
Addenbrookes Hospital (AH)	25	26
Leicester Royal Infirmary (LRI)	2	33
Royal Hampshire County Hospital (RCH)	3	4
Clatterbridge Hospital (CH)	2	13
University of Leeds (L)	6	19
Birmingham University Hospital (B)	-	9
Royal Hallamshire Hospital (RHH)	-	5
University Hospital Wales (W)	-	13
Bristol Southmead Hospital (BSH)	-	16

Table 2.1. Bacterial strains used in this study

Bacterial Strain	Source
70 *S. pneumoniae* strains	Clinical isolates were collected from centres throughout the UK between October 1995 and February 1996 by Bayer plc (Newbury, Berkshire) (see text for the list of centres)
231 *H. influenzae* strains	Clinical isolates were collected from centres throughout the UK between October 1995 and February 1996 by Bayer plc (Newbury, Berkshire) (see text for the list of centres)
10 penicillin-resistant *S. pneumoniae* strains: RIE-919, RIE-2905, RIE-6083, RIE-7184, RIE-8442, RIE-11825, RIE-12825, RIE-11080, RIE-13593, RIE-13678	Penicillin-resistant clinical isolates collected from the Royal Infirmary of Edinburgh (RIE) Hospital in 1996
R6 *S. pneumoniae* strain	Penicillin-susceptible, non-capsulated strain from Dr. P. V. Adrian, Department of Medical Microbiology, University of the Witwatersrand, Johannesburg, South Africa
Laboratory Standards:	
S. aureus NCTC 6571	National Collection of Type Cultures, London
E. coli NCTC 10418	
P. aeruginosa NCTC 10662	
S. pneumoniae NCTC 7465	Sensitive to all common antibiotics
H. influenzae NCTC 11931	

2.2. Storage of Cultures

Bacterial strains were stored at -70°C in Brain Heart Infusion broth (Oxoid, UK) with 50% glycerol. Strains were always subcultured from the stock on solid media and were not passaged.

2.3. Growth Media

All growth media were sterilised by autoclaving at 121°C for 15 min at 15 pounds per square inch. All media were obtained from Oxoid (Oxoid, UK) as dry powders, and prepared as single strength solutions as recommended by the manufacturer unless otherwise stated.

2.3.1. Blood Agar

S. pneumoniae strains were grown in Blood Agar plates. Columbia agar was prepared according to the manufacturer's instructions. The media was cooled below 55°C prior to the addition of 5% defibrinated horse blood and incubated at 37°C in a 5% CO_2 atmosphere overnight.

2.3.2. Chocolate Blood Agar

H. influenzae strains were grown in Chocolate Blood Agar plates. Columbia agar prepared was cooled to 65°C prior to the addition of 5% defibrinated horse blood and incubated at 37°C in a 5% CO_2 atmosphere overnight.

2.3.3. Brain Heart Infusion Broth

Bacterial strains were grown overnight in Brain Heart Infusion (BHI) broth (supplemented with 10 μg/ml Haemin and 10 μg/ml NAD, for *H. influenzae*) at 37°C in 5% CO_2.

2.4. Chemical Reagents

All chemicals and reagents were supplied by Sigma Chemical Co. (Poole, Dorset) unless otherwise stated.

CHAPTER THREE

Antimicrobial Susceptibility of *S. pneumoniae* and *H. influenzae* Clinical Isolates

3.1. Introduction

Antimicrobial resistance is an ever-present and ever-changing problem with organisms that cause serious diseases (Amyes, 1999). It is essential that there are well-controlled periodic surveys to monitor any changes in rates of resistance to the antimicrobial agents used traditionally as therapy for *S. pneumoniae* and *H. influenzae* and to determine whether resistance is developing to the newer agents that are currently active against these organisms.

An ideal antimicrobial agent should be effective, available in both oral and intravenous formulations, well distributed in the respiratory tract, well tolerated and safe, and inexpensive. Currently, there is no one drug that meets this profile. β-Lactams and macrolides are the most frequently used antibiotics in respiratory tract infections (Jacobs, 1997). However, macrolide resistance is increasing in *S. pneumoniae* and some macrolides have been found to have limited activity against penicillin-intermediate and penicillin-resistant strains of *S. pneumoniae* (Jacobs, 1997). Amoxycillin/clavulanate and cefuroxime axetil retain high levels of activity against β-lactamase producing strains of *H. influenzae* and penicillin-intermediate strains of *S. pneumoniae*.

The present study attempted to determine the prevalence of antimicrobial resistance among clinical isolates of *S. pneumoniae* and *H. influenzae* in the UK.

3.2. Materials & Methods

3.2.1. Bacterial strains

A total of 70 *S. pneumoniae* and 231 *H. influenzae* clinical isolates were obtained from centres throughout the UK. In addition, 10 penicillin-resistant *S. pneumoniae* isolates from the Royal Infirmary of Edinburgh were added to this study.

The following strains were also used for quality control and tested each time susceptibility testing was performed: *E. coli* NCTC 10418, *S. aureus* NCTC 6571, *P. aeruginosa* NCTC 10662, *S. pneumoniae* NCTC 7465 and *H. influenzae* NCTC 11931.

3.2.2. Antimicrobial Susceptibility Tests

Antimicrobial Agents

Antimicrobial agents were supplied as sterile powders of known potency from different suppliers (Table 3.1). Clavulanic acid was supplied by SmithKline Beecham. Drugs were stored as dry powders in darkness at 4°C. The antimicrobial solutions were freshly prepared with sterile MilliQ water when required.

Table. 3.1 Antimicrobial Agents

Antimicrobial Agent	Supplier
Benzyl Penicillin	Glaxo-Wellcome
Amoxycillin	Bencard
cefuroxime	Sigma Chemicals
Imipenem	Merck Sharp & Dohme
Meropenem	Zeneca Pharmaceutical
Cefaclor	Sigma Chemicals
Erythromycin	David Bull Laboratories
Tetracycline	Lederle Laboratories
Trimethoprim	Glaxo-Wellcome

Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibitory concentrations were determined by agar dilution as described in the standard guidelines of the British Society of Antimicrobial Chemotherapy (BSAC) (Philips *et al.*, 1991).

For the preparation of agar plates containing antimicrobial agents, doubling dilution of each antimicrobial agents were freshly made with sterile MilliQ water in Iso-Sensitest Agar plates supplemented with 5% defibrinated horse blood. The media containing antimicrobial agents were used within 24 h.

Bacterial strains were grown overnight in Iso-Sensitest Broth (Oxoid, UK) (supplemented with 10 μg/ml Haemin and NAD, for *H. influenzae*) at 37°C with continuous shaking. Plates containing antimicrobial agents were inoculated by transferring 2 μl volume of the freshly diluted bacterial suspension (10^4 cfu/ml) from overnight culture to the surface of the agar with a mulipoint inoculator (Denly, Billinghurst, UK). A plate with no added antibiotic was inoculated as a positive control. All plates were incubated at 37°C in 5% $C0_2$ for 18-24 h. The lowest concentration of the antimicrobial agents to inhibit all visible growth was determined as the MIC (mg/L).

3.2.3. Investigation of the Effect of Incubation Conditions on Trimethoprim MIC Results

i) Agar Dilution Method

Seventy pneumococcal strains were re-examined to determine their susceptibility to trimethoprim by an agar dilution method (as described above). MIC plates containing trimethoprim were incubated aerobically and in CO_2 at 37°C for 18-24 h and the MIC recorded.

ii) The E-test Method

High trimethoprim MICs against pneumococcal strains were further investigated using the E-test (Cambridge Diagnostics Services LTD, UK). Four Iso-Sensitest agar plates supplemented with 5% horse blood were prepared. Two plates were incubated at 37°C aerobically (A_1 & A_2) and the other two were incubated at 37°C in 5% CO_2 (C_1 & C_2) without inoculating the pneumococcal strain. At the same time, the test pneumococcal strain (with trimethoprim MIC of 4 mg/L) was grown overnight in Iso-Sensitest Broth at 37°C with continuous shaking. After overnight incubation, loopfuls of diluted bacterial suspension (10^4 cfu/ml) were streaked into the surface of the four pre-incubated Iso-Sensitest agar plates supplemented with 5% horse blood. The E-test trimethoprim strips were applied to each plate. Plates A_1 and C_1 were re-incubated at 37°C aerobically, and plates A_2 and C_2 were re-incubated at 37°C in 5% CO_2 for 18-24 h. After overnight incubation the MIC results were recorded.

3.3. Results

3.3.1. Prevalence of Antimicrobial Resistance in *S. pneumoniae*

Of the 70 pneumococcal isolates tested, 8 (11.4%) strains were resistant to penicillin (MIC ≥0.12 mg/L). Two of these showed intermediate resistance (MIC 0.12-1 mg/L) to penicillin and 6 were resistant (MIC >1 mg/L). The rate of resistance to other antibiotics were as follows: 7.1% amoxycillin, 4.3% co-amoxiclav, 12.9% erythromycin, 10% tetracycline, 10% cefuroxime and interestingly 94.2% of the isolates were resistant to trimethoprim. All the pneumococcal isolates were susceptible to imipenem and meropenem (Table 3.2).

The other 10 penicillin-resistant *S. pneumoniae* isolates from the Royal Infirmary of Edinburgh hospital were added to the 8 penicillin-resistant *S. pneumoniae* isolates from the UK. These 18 penicillin resistant *S. pneumoniae* isolates from UK were further examined. In this new group, 8 isolates were intermediate resistant to penicillin and the remaining 10 isolates were highly resistant.

Fully susceptible strains of *S. pneumoniae* have MICs of penicillin of 0.008 to 0.06 mg/L, and strains exhibit a normal distribution around an MIC of 0.016 mg/L (Figure 3.1). Intermediate strains have penicillin MICs of between 0.12 and 1 mg/L, while strains with MICs over 1 mg/L are considered resistant. In contrast to penicillin, strains tended to be either highly susceptible or highly resistant to the macrolides (Figure 3.2.) when a susceptible breakpoint of ≤0.25 mg/L was used for erythromycin (Jacobs & Appelbaum, 1995).

The pattern of multi-resistance observed is summarised in Table 3.3. The results show that there is a significant relationship between penicillin-resistance and cross-resistance to tetracycline, trimethoprim and erythromycin.

Table 3.2. Prevalence of antimicrobial resistance among 70 clinical isolates of *S. pneumoniae* collected from the UK

Antimicrobial Agent (Range tested in mg/L)	Resistant breakpoint* (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	% (no.) Resistant	Range (mg/L)
Penicillin (0.008-8)	≥0.12	0.008	0.016	11.4 (8)	0.008-8
Amoxycillin (0.008-8)	≥1	0.008	0.016	7.1 (5)	0.008-2
Co-amoxiclav (0.008-8)	≥1	0.008	0.016	4.3 (3)	0.008-2
Cefuroxime (0.008-8)	≥1	0.008	0.016	10 (7)	0.004-4
Imipenem (0.004-16)	≥4	0.004	0.004	0	0.004-0.12
Meropenem (0.004-16)	≥4	0.004	0.004	0	0.004-0.25
Tetracycline (0.03-128)	≥1	0.12	0.25	10 (7)	0.03-128
Erythromycin (0.06-8)	≥0.5	0.06	0.12	12.9 (9)	0.06-16
Trimethoprim (0.12-128)	≥0.5	2	8	94.2 (66)	0.12-128

* Figures adopted from Philips *et al.* (1991).

Figure 3.1. Distribution of MICs of penicillin for 70 *S. pneumoniae* clinical isolates

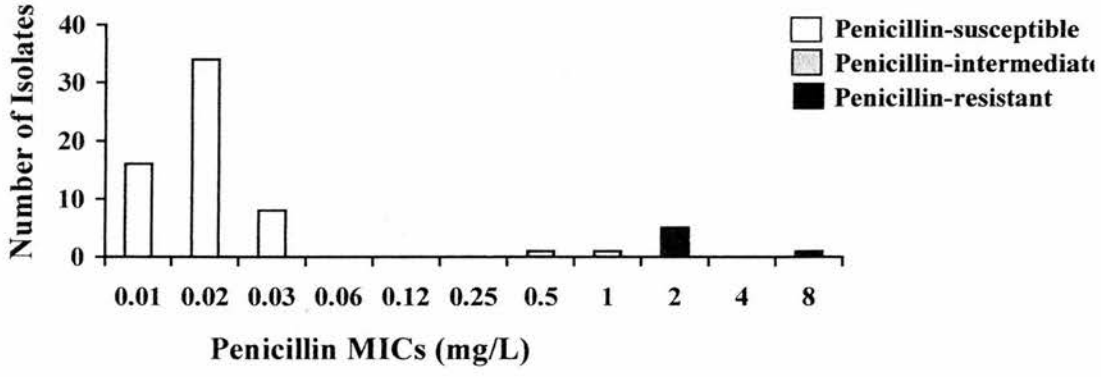


Figure 3.2. Distribution of MICs of erythromycin for 70 *S. pneumoniae* clinical isolates

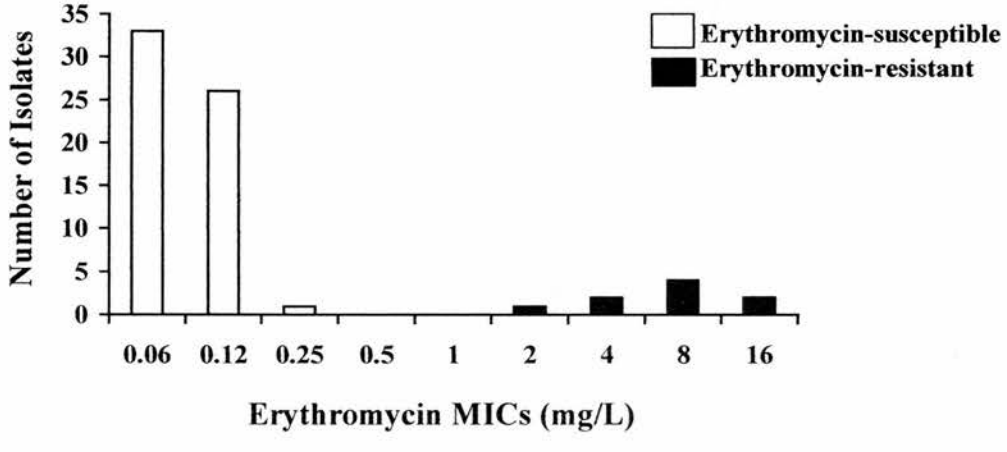


Table 3.3. Pattern of resistance in three groups of *S. pneumoniae* strains delineated by penicillin susceptibility

Also Resistant to	Penicillin Susceptibility		
	% (no.) Susceptible (< 0.12 mg/L) *n=62*	% (no.) Intermediate (0.12-1 mg/L) *n=8*	% (no.) Resistant (> 1 mg/L) *n=10*
Tetracycline	8 (5)	62.5 (5)	30 (3)
Erythromycin	8 (5)	62.5 (5)	50 (5)
Trimethoprim	32.3 (20)	62.5 (5)	80 (8)

3.3.2. Effect of Incubation Conditions on Trimethoprim MICs

i) Trimethoprim MIC Results by Agar Dilution Method

Trimethoprim MICs were reexamined for the 70 pneumococcal strains to determine the effect of air and CO_2 incubation in trimethoprim susceptibility. MIC plates incubated in 5% CO_2 gave high MIC results. However, there was a substantial decrease in the MICs of trimethoprim when plates were incubated aerobically in which 67.1% (47) of isolates were resistant to trimethoprim compared to 94.2% (66) of isolates were resistant when plates were incubated in 5% CO_2.

ii) Trimethoprim MIC Results by the E-test

The high trimethoprim MIC for pneumococcal strains when the MIC plates were incubated in 5% CO_2, was further investigated using the E-test (Table 3.4). Plates A_1 and C_1 showed MIC of 4 mg/L for trimethoprim. On the other hand, plates A_2 and C_2 showed high MIC results (MIC >32 mg/L). The results suggested that the microaerophilic condition rather than changes in pH of the medium antagonise trimethoprim and that preincubation had no effect on trimethoprim MIC results.

Table 3.4. The effect of incubation condition in trimethoprim MIC by the E-test

Agar Plate	Iso-Sensitest agar plates supplemented with 5% defibrinated horse blood				
	Pre-incubated overnight initially without pneumococcal strain in		Re-incubated overnight with pneumococcal strain and trimethoprim E-test strip in		Trimethoprim MIC (mg/L)
	Air	CO_2	Air	CO_2	
A_1	x		x		4
A_2	x			x	>32
C_1		x	x		4
C_2		x		x	>32

3.3.3. Prevalence of Antimicrobial Resistance in *H. influenzae*

Of the 231 *H. influenzae* isolates tested, 46 (20%) strains were resistant to amoxycillin (MIC ≥1 mg/L) and 9 (3.9%) isolates were also resistant to co-amoxiclav (MIC ≥1 mg/L).

The rate of resistance to other antibiotics was as follows: 97% erythromycin, 3.9% tetracycline, 5.2% cefuroxime and 9.5% of the isolates were resistant to trimethoprim. All the *H. influenzae* isolates were susceptible to ciprofloxacin (Table 3.5).

Table 3.5. Prevalence of antimicrobial resistance among 231 clinical isolates of *H. influenzae* collected from the UK

Antimicrobial Agent (Range tested in mg/L)	Resistant breakpoint* (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	% (no.) Resistant	Range (mg/L)
Amoxycillin (0.03-128)	≥1	0.05	4	20 (46)	0.03-64
Co-amoxiclav (0.03-16)	≥1	0.5	1	3.9 (9)	0.03-4
Cefuroxime (0.25-16)	≥1	0.5	1	5.2 (12)	0.25-4
Cefaclor (0.12-128)	≥8	4	16	10 (23)	0.5-32
Tetracycline (0.06-128)	≥1	0.5	1	3.9 (9)	0.016-8
Erythromycin (0.12-128)	≥0.5	4	16	97 (224)	0.12-32
Trimethoprim (0.016-128)	≥0.5	0.12	0.5	9.5 (22)	0.016-16
Ciprofloxacin (0.004-5)	≥1	0.008	0.016	0 (0)	0.008-0.5

* Figures adopted from Philips *et al.*, (1991).

3.4. Discussion

S. pneumoniae and *H. influenzae* are responsible for systematic as well as upper and lower respiratory tract infections. In addition, *S. pneumoniae* and *H. influenzae* are frequently implicated in bacterial meningitis (Nazareth *et al.*, 1992; Schelch *et al.*, 1985). Treatment of these potentially serious infections has become problematic because of increasing global antimicrobial resistance (Gruneberg & Felmingham, 1996). Since the prevalence and resistance patterns continue to change, ongoing surveillance is required.

The prevalence of penicillin-resistant pneumococci is increasing worldwide, and is greater than 20% in many parts of the world (Dowson *et al.*, 1994), similarly, macrolide-resistant strains are usually in the range of 10 to 20% (Appelbaum, 1992). In *H. influenzae,* resistance rates to certain β-lactams (e.g. amoxycillin-clavulanate and cefixime) are low, whereas resistant rates to cefaclor and cefprozil are higher (>5%) (Jones *et al.*, 1997). The use of macrolides against *H. influenzae* is limited by their marginal activity. Azithromycin is the most potent macrolide compared with erythromycin and clarithromycin. No high-level resistance to the macrolides has been detected in *H. influenzae*.

Until recently the UK was considered to have a low prevalence of penicillin-resistant pneumococci. As recently as 1990, 100% of 7255 strains of pneumococci from centres across the UK were found to be penicillin susceptible (Spencer *et al.*, 1990). However, there have now been several reports of significant and rising levels of resistance nationwide (Goldsmith *et al.*, 1997).

The results indicated that the UK has fewer problems with regards to antibiotic resistance in *S. pneumoniae* and *H. influenzae* than many other countries. Nevertheless, there is a continuing increase in resistance to certain antibiotics and therapy with these antibiotics would be expected to encounter more problems with drug resistance than a decade ago.

CHAPTER FOUR

Molecular Typing and Analysis of Penicillin-Binding Protein Alterations in Penicillin-Resistant Clinical Isolates of *S. pneumoniae*

4.1. Introduction

Infections caused by *S. pneumoniae* contribute significantly to morbidity and mortality world-wide (Klugman, 1990). Although penicillin was the treatment of choice for *S. pneumoniae* infections, the development of penicillin resistance has complicated treatment strategies and resulted in alternative agents being used. Resistance to penicillin and other β-lactam antibiotics in *S. pneumoniae* results from alteration of penicillin binding proteins (PBPs) 1a, 1b, 2x, 2a and 2b which reduce β-lactam affinity (Hakenbeck *et al.*, 1980). The overall resistance of an individual strain is dependent on the pattern of PBP alterations. The nucleotide sequences of genes coding for the native and altered PBP (2b, 2x and 1a) of both penicillin-resistant and penicillin-susceptible *S. pneumoniae* have been reported (Dowson *et al.*, 1989a; Laible *et al.*, 1991, Martin *et al.*, 1992).

Penicillin resistance in *S. pneumoniae* results in the use of alternative agents and it has been observed that the emergence of penicillin resistance in *S. pneumoniae* is often followed by an increase in strains resistant to other antibiotics leading to multidrug resistance which is defined as resistance to three or more different classes of antibiotics (Appelbaum, 1992). There are distinct differences in the incidence of penicillin resistance in *S. pneumoniae* in different countries. Within Europe the highest rates have been found in Spain and Hungary with levels in excess of 50% reported

(Marton *et al.*, 1991). Until recently penicillin resistance in *S. pneumoniae* in UK was unusual, however, the situation is changing and reports of penicillin resistance in *S. pneumoniae* have been made from a number of centres (Goldsmith *et al.*, 1997). The factors that contribute to the overall levels of penicillin resistance are complex: a correlation between penicillin resistance and the usage of penicillin has been demonstrated (Baquero, 1996) but equally the importation of resistant isolates from other areas followed by the clonal spread of these organisms has been shown to be important in other countries (Muñoz *et al.*, 1992; Soares *et al.*, 1993). Recently the evolution of penicillin resistance in *S. pneumoniae* has been modelled, predicting a slow emerging phase of resistance development followed by exponential increases in resistance over a ten year period to a stationary phase level of around 50% resistance (Baquero, 1996). It has been speculated that the United Kingdom may be at the start of the exponential growth phase of penicillin resistant *S. pneumoniae* (Goldsmith *et al.*, 1997).

It is essential therefore that the emergence of penicillin resistance in *S. pneumoniae* in the UK be monitored closely. In this study, clinical respiratory isolates of penicillin-resistant *S. pneumoniae* from centres in the UK have been molecularly typed by pulsed-field gel electrophoresis (PFGE) and screened for alterations, in the genes encoding PBP 2B, 2X and 1A by the polymerase chain reaction (PCR). Also restriction by *Hin*fI of entire *pbp2x, 2b* and *1a* genes amplified by PCR have been used. This data will allow us to determine if increasing penicillin resistance in the UK results from clonal spread of particular strains or is emerging independently.

4.2. Materials & Methods

4.2.1. Bacterial Strains

Eighteen penicillin-resistant *S. pneumoniae* clinical isolates with penicillin G MICs of ≥ 0.12 mg/L were used in this study (See section 3.3.1). All isolates were grown on a Columbia agar supplemented with horse blood (5%) at 37° C in 5% CO_2.

4.2.2. Capsular Serotyping

S. pneumoniae penicillin-resistant isolates were serotyped on the basis of capsular swelling (Quellung reaction) with type-specific antisera (Statens Serum Institute, Copenhagen) by the Central Public Health Laboratory Service (PHLS, Colindale, London).

4.2.3. Pulsed-Field Gel Electrophoresis (PFGE)

Preparation of Intact Chromosomal DNA in Agarose Plugs

The preparation of intact chromosomal DNA in agarose plugs was by the method of Hall *et al.* (1996) with some modifications. *S. pneumoniae* strains were inoculated into Brain Heart Infusion broth (BHI) and were grown to an optical density of 0.3 to 0.5 at 560 nm in 37°C incubator with 5-10% CO_2 overnight. The cells were centrifuged at 4°C at 3,000 rpm in a centrifuge for 10 min. The cell pellets were resuspended in 0.5 ml TN buffer [10 mM Tris-HCl (pH 7.6), 1 M NaCl], recentrifuged, resuspended finally in fresh TN buffer and held at 37°C. Each 0.5 ml cell suspension was then mixed with 0.5 ml of molten 2% low-melt preparative grade agarose (Bio-Rad, UK) in distilled water. The mixture was micropipetted immediately

into the wells of a perspex plug mould (Bio-Rad, UK) and cooled to 4°C to allow the agarose to set. Each set of plugs was then ejected from the mould into bijou bottles containing 2 ml of lysis buffer [6 mM Tris-HCl (pH 7.6), 1 M NaCl, 100 mM disodium EDTA, 0.5% polyxythylene 20 cetyl ether, 0.2% sodium deoxycholate, 0.5% *n*-lauroylsarcosine, 20 μg/ml ribonuclease A, and 100 μg/ml lysozyme] and the bottles were incubated overnight at 37°C. The lysis buffer was then replaced with 2 ml of EL buffer [0.5 M disodium EDTA (pH 9.0), 1% *n*-lauroylsarcosine, 50 μg/ml proteinase K] and incubated at 55°C for 48 h. At the end of this period, the EL buffer was replaced with 2 ml of TE buffer [10 mM Tris-HCl, 0.1 mM disodium EDTA] and incubated at 4°C for 30 min. This wash step was repeated twice with fresh TE buffer for a minimum of 30 min each. The plugs were then stored at 4°C in TE buffer until required.

Digestion of Chromosomal DNA in Agarose Plugs

Generally, digests were performed with DNA contained in about one-third of a complete plug. Each plug portion was transferred to a sterile microcentrifuge tube and equilibrated at 4°C in 100 μl of the *Sma*I digestion buffer (Promega, UK). After 1 h, the buffer was replaced with 100 μl of fresh digestion buffer and 12 U of *Sma*I (Promega, UK) was added. After 4-6 h digestion at 37°C, the buffer-enzyme mixture was replaced with 100 μl of inactivation buffer [0.5 M EDTA (pH 9.0), 1% *n*-lauroylsarcosine] and kept at 4°C until ready for electrophoresis.

Electrophoresis

DNA macrorestriction fragments were separated on 1% agarose gel by PFGE employing a CHEF-DR II system (Bio-Rad, UK) at 6 V/cm for 18 h with a switching time of 1-15 sec at 12°C. The gels were stained with 1 μg/ml of ethidium bromide and were destained in water for 1h before photography.

Gelcompar Software

The PFGE patterns were analysed by Gelcompar software (Applied Math, Kortrijk, Belgium). The PFGE patterns were compared by the UPGMA (unweighted pair group method with arithmetic averages) clustering method employing the Dice coefficient (Shi *et al.*, 1996). A tolerance in the band position of 1.2% was applied during the comparison of PFGE fingerprinting patterns.

4.2.4. Isolation of Chromosomal DNA

S. pneumoniae strains were cultivated on Columbia agar supplemented with 5% horse blood and incubated in 5-10% CO_2 at 37°C overnight. Colonies were emulsified in 50 µl sterile MilliQ water in a microcentrifuge tube and boiled for 5 min; the supernatant containing DNA was used for PCR analysis.

4.2.5. DNA Amplification by Polymerase Chain Reaction

The PCR protocol was a modification of that used by Jalal *et al* (1997). The primers used (Table 4.1) were supplied high-pressure liquid chromatography (HPLC) purified by Oswel DNA Services (Southhampton, UK). Primers derived from sequences encoding the penicillin-binding domain of PBPs were used to amplify susceptible genotypes of *pbp2b* and *2x*, and resistant genotype of *pbp 1a* genes.

Table 4.1. Primers sequence used in PCR to amplify part of *pbp2b*, *2x* and *1a* genes in *S. pneumoniae*

Name	Sequence (5`___3`)
pbp2b	ACTCAGGCTTACGGTTCATT
pbp2b`	ACGAGGAGCCACACGAACAC
pbp2x	GTCATGCTGGAGCCTAAATT
pbp2x`	AACCCGACTAGATAACCACC
pbp1a	AGGTCGGTCCTAGATAGAGCT
pbp1a`	GAGCTACATAGCCAGTGTCTC

All the PCR reagents were supplied by Advanced Biotechnologies Ltd., UK. The optimal reaction mix included 0.5 µl of *Taq* DNA Polymerase (5 U/µl), 10 µl of 10x Reaction buffer [200 mM $(NH_4)_2SO_4$, 750 Mm Tris-HCl (pH 8.8), 0.1% Tween®20], 5 µl dNTP Mix (20 mM), 3 µl $MgCl_2$ (25 mM), 1 µl primers Mix (10 pmole/µl), 5 µl of DNA template, and sterilised MilliQ water to a final volume of 100 µl. This was overlaid with sterile mineral oil and processed on Techne Thermal Cycler (Techne Cambridge Ltd., UK). The optimal PCR cycling conditions were as follows: 1 cycle of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72° C for 2 min followed by a final cycle of 72° C for 7 min. Two controls were included in each PCR run: *S. pneumoniae* R6 control strain and a negative control where DNA was replaced by an equal amount of sterilised MilliQ water. PCR products were analysed by electrophoresis through a 1.8 % agarose-ethidium bromide-containing gel with 100 bp DNA ladder (GibcoBRL Life technologies) as marker and bands visualised with UV transillumination.

4.2.6. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Amplification of the Entire *pbp2b*, *2x* and *1a* Genes by Polymerase Chain Reaction

The PCR protocol was a modification of that used by Gillespie *et al* (1997). The primers used (Table 4.2) were supplied high-pressure liquid chromatography (HPLC) purified by Oswel DNA Services (Southhampton, UK). Primers used were designed to amplify the entire *pbp2x*, *2b* and *1a* genes.

Table 4.2. Primers sequence used in PCR to amplify the entire *pbp2b*, *2x* and *1a* genes in *S. pneumoniae*

Name	Sequence (5`___3`)
pbp2b	GATCCTCTAAATGATTCTCAGGTGG
pbp2b`	CAATTAGCTTAGCAATAGGTGTTGG
pbp2x	CGTGGGACTATTTATGACCGAAATGG
pbp2x`	AATTCCAGCACTGATGGAAATAAACATATTA
pbp1a	CGGCATTCGATTTGATT
pbp1a`	GATGTCTTCTCAGGCTTTTG

The optimal reaction mixtures for PCR were as described above (see section 4.2.4). The cycling conditions were as follows: 95°C for 5 min, followed by 1 min of denaturation at 94° C, 2 min of annealing at 55° C, and 3 min of extension at 72° C for 30 cycles and a final extension for 7 min at 72° C in the same thermocycler with appropriate controls as described above.

Restriction Fragments Length Polymorphism with *Hin*fI

Restriction of the *pbp2x, 2b* and *1a* PCR products was performed using *Hin*fI (Promega, UK). The digestion mixture consisted of 1 µl of *Hin*fI enzyme (10 U/µl) in 10 mM Tris-HCl (pH 7.5), 60 mM $NaCl_2$, 7 mM $MgCl_2$, 0.1 mg bovine serum albumin per liter in 50 µl volume. A total of 20 µl of PCR product was added to 2 µl of buffer and 1 µl of *Hin*fI enzyme. Digestion proceeded for 4 h at 37°C in waterbath, and the products were run on a 1.8% agarose gel as described above.

4.3. Results

4.3.1. Capsular Serotyping

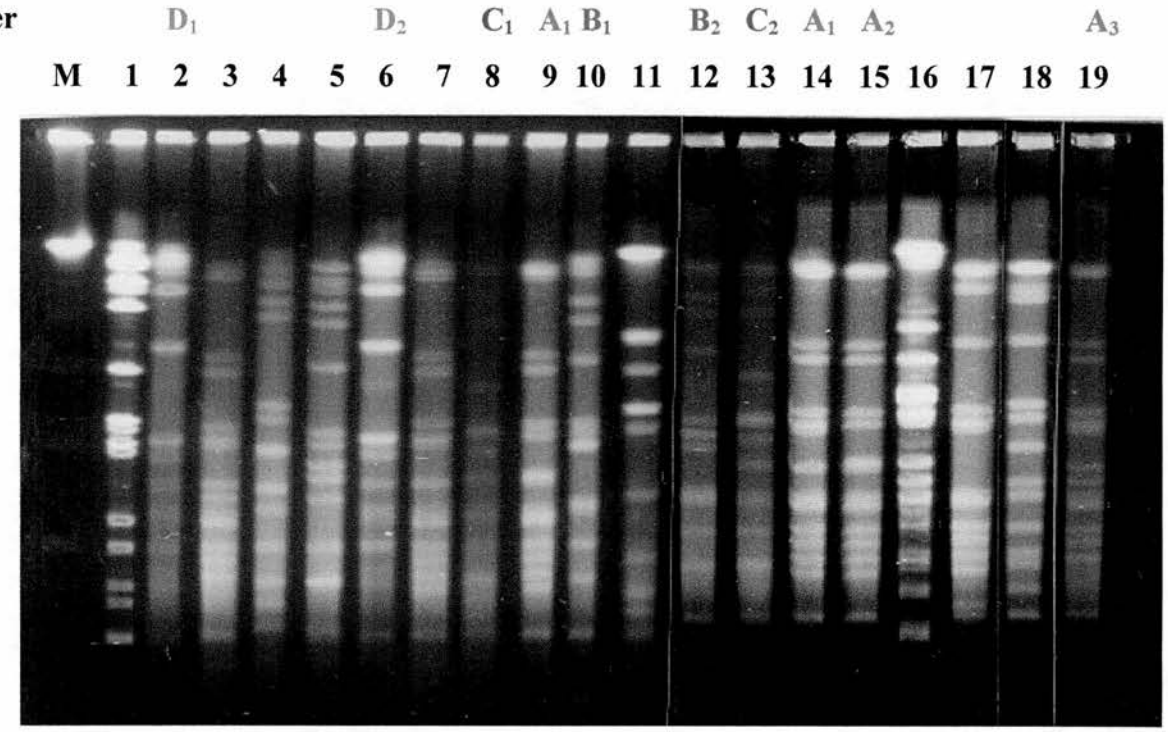
Of the 18 penicillin-resistant clinical isolates tested: nine isolates were serotype 9, four isolates were serotype 6, two isolates were serotype 19, two isolates were serotype 23 and one isolate was serotype 29. Interestingly, 7/9 isolates that exhibited serotype 9 had a high MIC of penicillin (≥2 mg/L).

4.3.2. Pulsed-Field Gel Electrophoresis (PFGE)

The PFGE patterns of 18 isolates from the UK are shown in Figure 4.1. These patterns were classified into groups alphabetically. PFGE fingerprinting of the 18 isolates analysed by Gelcompar revealed that only 2 of the isolates were identical. Taking a cut-off value of 80% similarity produced 4 clusters of isolates (A to D) within 10 strains from the total of 18 strains examined. The remaining 8 isolates were unrelated and demonstrated 8 distinct types (E to L).

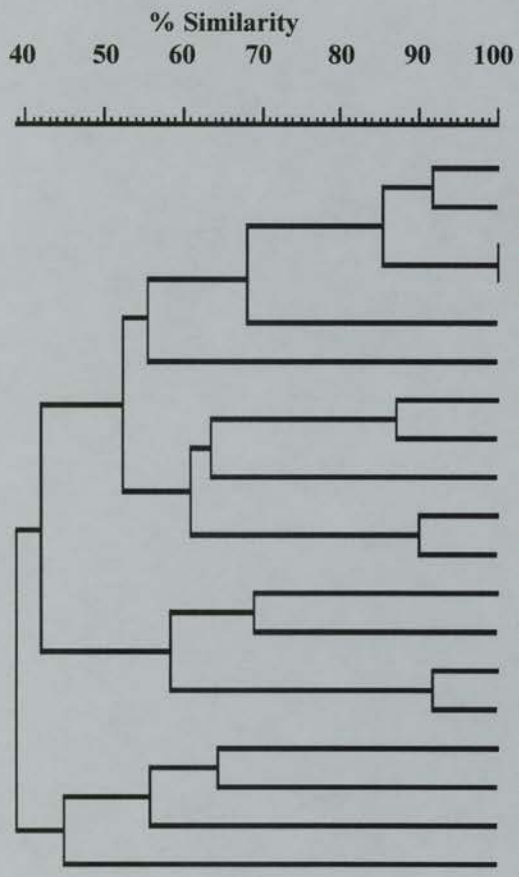
A dendrogram was constructed to show the degree of relatedness among the strains of clusters from the UK (Figure 4.2.). Cluster A included four isolates. Three genetically related subtypes were identified among these four isolates. The two identical isolates were of subtype A_1 (RIE-11080 & BRI-251). The remaining two isolates were subtypes A_2 & A_3 (NMH-285 & D-774, respectively). Cluster B included two isolates (RIE-13593 & NMH-158) and were subtyped B_1 and B_2, respectively. Cluster C included two isolates (RIE-12825 & BRI-249) and were subtyped C_1 and C_2, respectively. Cluster D also included two isolates (RIE-919 & RIE-8442) and were subtyped D_1 and D_2, respectively.

Figure 4.1. Pulsed-field gel electrophoresis of *Sma*I digested genomic DNA from 18 penicillin-resistant *S. pneumoniae* isolates from the UK



Lane M	lambda DNA ladder marker
Lane 1	R6 penicillin-susceptible *S. pneumoniae* strain
Lane 2-19	penicillin-resistant *S. pneumoniae* strain RIE-919, RIE-2905, RIE-6083, RIE-7184, RIE-8442, RIE-11825, RIE-12825, RIE-11080, RIE-13593, RIE-13678, NMH-158, BRI-249, BRI-251, NMH-285, WH-563, WH-565, D-680 & D-774.

Figure 4.2. Dendrogram illustrating the relatedness of 18 penicillin-resistant *S. pneumoniae* Isolates from the UK



Lane	Strain	Penicillin MIC (mg/L)	Serotype	PFGE	RFLP PBP 2b	RFLP PBP 2x
15	NMH-285	2	9	A_2	II_1	II_1
19	D-774	2	9	A_3	II_1	II_1
9	RIE-11080	0.25	6	A_1	II_2	I_1
14	BRI-251	2	9	A_1	II_1	II_1
7	RIE-11825	2	9	E	I_1	I_1
3	RIE-2905	1	19	F	I_2	I_1
10	RIE-13593	2	23	B_1	II_1	II_1
12	NMH-158	8	9	B_2	II_2	II_1
17	WH-565	2	9	J	II_1	II_1
8	RIE-12825	2	9	C_1	II_1	I_2
13	BRI-249	2	6	C_2	II_1	II_1
1	R6 Control	0.008	-	-	I_1	I_1
5	RIE-7184	0.25	19	K	II_2	I_2
2	RIE-919	0.12	6	D_1	I_1	I_1
6	RIE-8442	0.12	6	D_2	I_3	I_1
16	WH-563	1	9	I	II_1	II_1
18	D-680	0.5	9	L	II_1	II_1
4	RIE-6083	0.25	29	H	I_1	I_1
11	RIE-13678	2	23	G	II_1	II_1

The scale measures similarity values. Taking a cut off value of 80% similarity, four clusters within 10 isolates were identified (Typed A to D): Cluster type A were classified into three subtypes (A_1, A_2 & A_3), Cluster type B were classified into two subtypes (B_1 & B_2), Cluster type C were classified into two subtypes (C_1 & C_2), & Cluster type D were classified into two subtypes (D_1 & D_2).
The remaining 8 isolates demonstrated 10 distinct types (E to L).

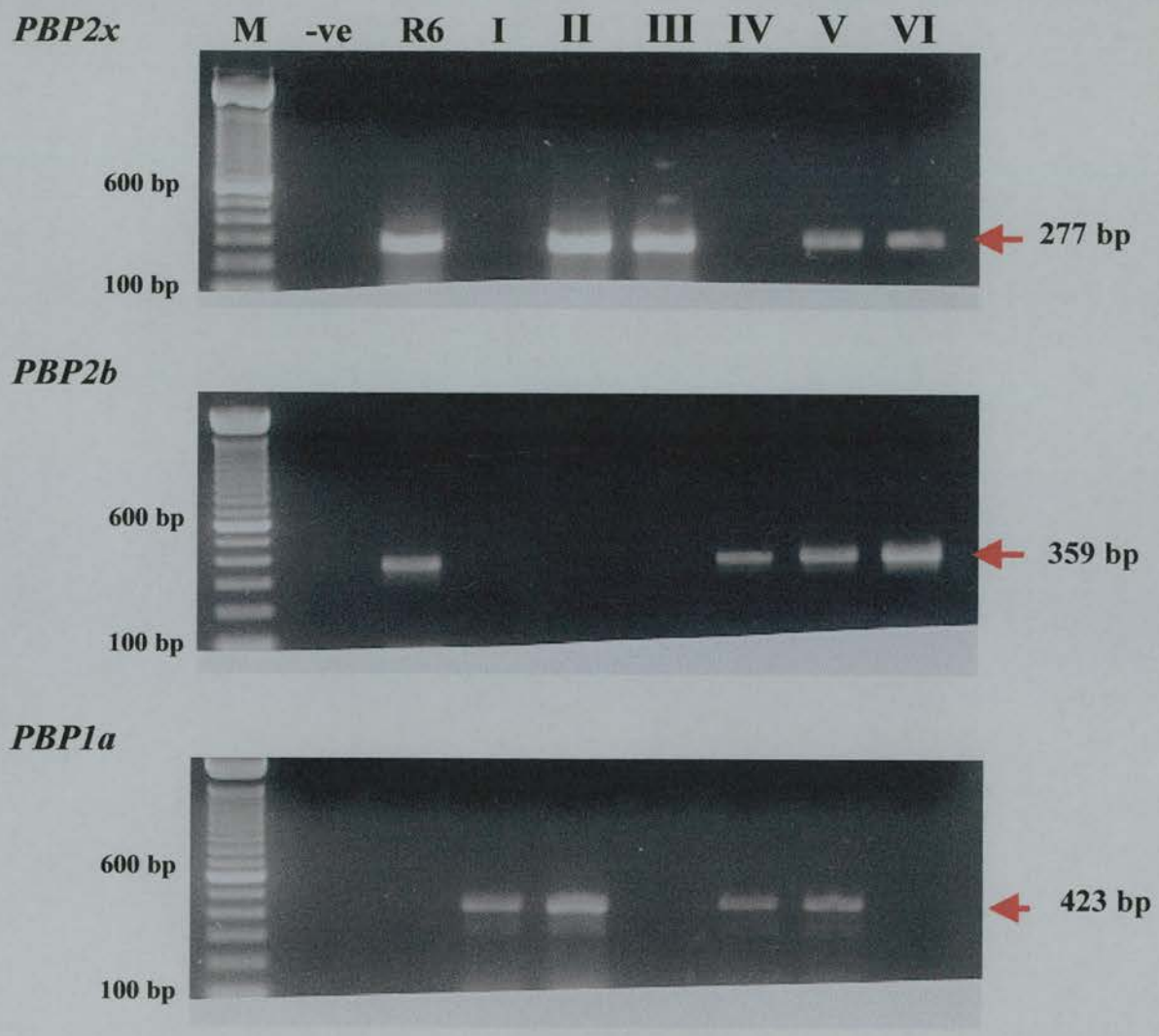
4.3.3. PCR Amplified *pbp*2x, *2b* and *1a* genes

Chromosomal DNA from *S. pneumoniae* strains was used as a template for PCR to amplify susceptible genotypes of *pbp2b* and *2x*, and resistant genotype of *pbp1a* genes. Oligonucleotide primer pairs allowed amplification and isolation of 277, 359 and 423 bp of *pbp2x, 2b* and *1a* products, respectively (Figure 4.3).

The isolates tested displayed six patterns of *pbp* genes when screened by PCR (Table 4.3 & Figure 4.3) and a Roman numeral was assigned to each pattern. Pattern type I consisted of alterations in the genes encoding PBP 2b, 2x and 1a and was detected in 6 isolates all of which were highly resistant to penicillin (MIC 2 mg/L). Four isolates with MICs of penicillin ranging from 1-8 mg/L showed a different pattern termed type II with alterations at *pbp* genes *2b* and *1a* only. Three isolates with intermediate levels of penicillin resistance (MIC 0.12-0.25 mg/L) had alterations in only *pbp* gene *2b* (Pattern III). Two further isolates with intermediate penicillin resistance had alterations in *pbp2x* and *1a* (Pattern IV). Pattern V was shown by only a single isolate with a high level of penicillin resistance (MIC 2 mg/L) and an alteration at only in *pbp1a*. Finally two isolates with intermediate levels of penicillin resistance (MIC 0.12-0.25 mg/L) had no detectable change in the genes encoding *pbp2b, 2x* or *1a* (Pattern VI).

Figure 4.3. Agarose gel electrophoresis of *pbp2x, 2b* and *1a* amplified PCR product

	PCR Patterns					
	I	II	III	IV	V	VI
PBP2x	+	-	-	+	-	-
PBP2b	+	+	+	-	-	-
PBP1a	+	+	-	+	+	-



Lane M, 100 bp lambda DNA ladder marker
Lane –ve, negative control (without template DNA)
Lane R6, R6 penicillin-susceptible *S. pneumoniae* strain
Lanes I-VI, PCR Patterns
(+) & (-), Presence and absence of altered PBPs, respectively.

Table 4.3. Relationship of PBP alterations in 18 penicillin-resistant *S. pneumoniae* isolates from the UK

Strain	Penicillin MIC mg/L	PCR Pattern	PBP genotype		
			2X	2B	1A
RIE-11825	2	I	+	+	+
RIE-12825	2		+	+	+
BRI-249	2		+	+	+
BRI-251	2		+	+	+
NMH-285	2		+	+	+
D-774	2		+	+	+
RIE-2905	1	II	-	+	+
RIE-13593	2		-	+	+
RIE-13678	2		-	+	+
NMH-158	8		-	+	+
RIE-919	0.12	III	-	+	-
RIE-6083	0.25		-	+	-
RIE-8442	0.12		-	+	-
RIE-11080	0.25	IV	+	-	+
WH-563	1		+	-	+
WH-565	2	V	-	-	+
RIE-7184	0.25	VI	-	-	-
D-680	0.5		-	-	-

PFGE Pulsed-field gel electrophoresis, **PCR** Polymerase chain reaction, **PBP** Penicillin binding protein, (+)altered pattern, (-)not altered pattern.

4.3.4. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Chromosomal DNA from *S. pneumoniae* strains was used as a template for PCR to amplify the entire *pbp2x, pbp2b* and *1a* genes. Oligonucleotide primer pairs allowed amplification and isolation of 1.5 bp *pbp2b* products and 2.0 bp *pbp 2x* products (Figure 4.4). However, the *pbp1a* gene did not amplify.

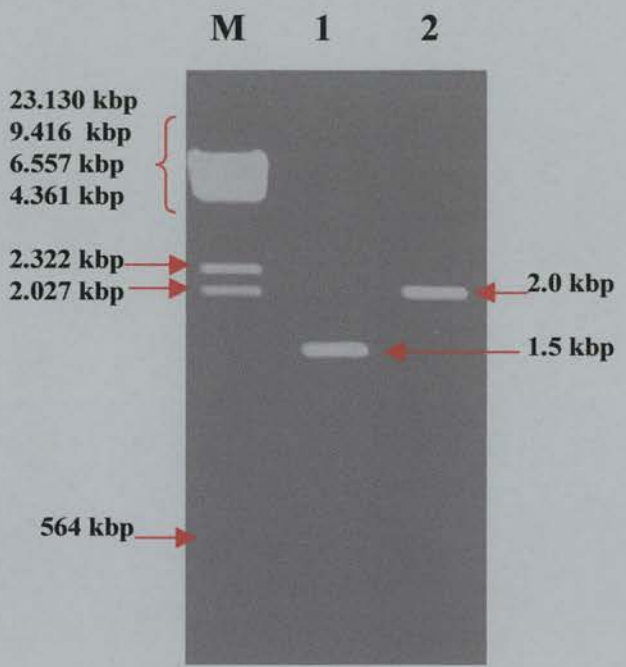
The restriction patterns of the *pbp2b* and *2x* genes of all strains with an MIC of penicillin of 1 mg/L or above were different from the penicillin-susceptible strain R6 (Figures 4.5). In addition, strains with an MIC less than 1 mg/L showed heterogeneous patterns of restriction.

All strains with an MIC of penicillin of 1 mg/L or above had the same restriction pattern for both *pbp2b* and *2x*, except strains WH-563 and BRI-249. All strains with an MIC for penicillin of less than 1 mg/L had the same pattern as penicillin-susceptible strain R6 for both *pbp2b* and *2x*, except strains RIE-11080, RIE-2905 and WH-563. Interestingly, the same pattern of restriction of *pbp2b* and *2x* genes was observed in two isolates with serotype 23, and 7 of the 9 isolates with serotype 9.

S. pneumoniae strains with an MIC of penicillin of 1 mg/L or above exhibited considerable homogeneity in *pbp2b* and *2x* RFLP. However, strains with an MIC less than 1 mg/L gave a more heterogeneous pattern.

The relationship of RFLP patterns, PFGE patterns, capsular serotypes and MICs of 18 penicillin-resistant *S. pneumoniae* clinical isolates from the UK is shown in Table 4.4.

Figure 4.4. Agarose gel electrophoresis of the entire *pbp2x* and *2b* genes PCR products



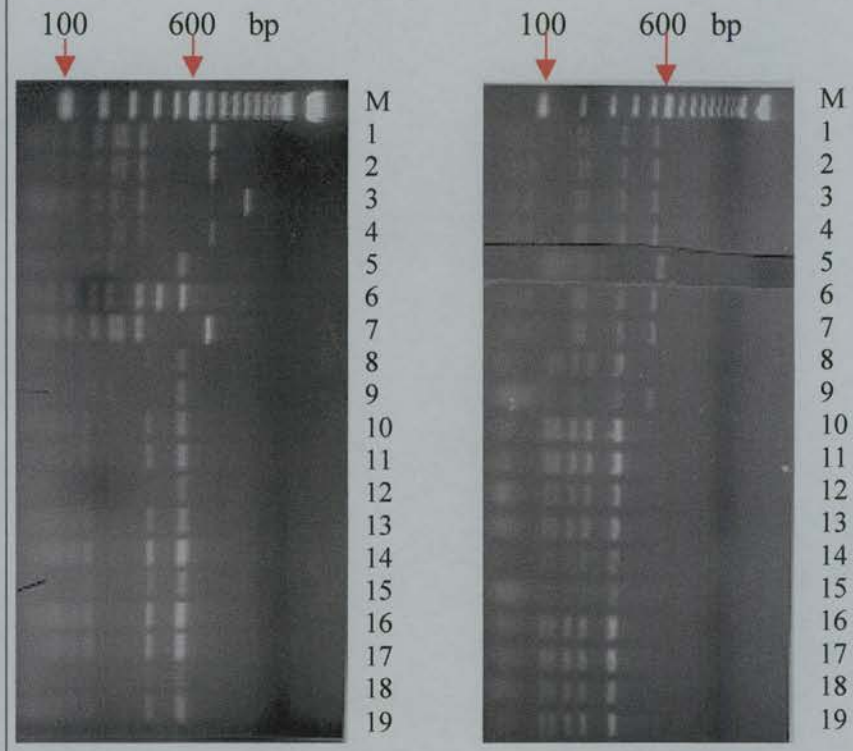
Lane M λ DNA/*Hin*dIII molecular size marker

Lane 1 *pbp2b* PCR product

Lane 2 *pbp2x* PCR product

Figure 4.5. Relationship of penicillin resistance and RFLP in 18 penicillin-resistant *S. pneumoniae* isolates from the UK

Lane	Strain	Penicillin MIC (mg/L)		RFLP of PBP		Restriction Fragment Length Polymorphism (RFLP)
		Interm. 0.12-1	High ≥ 2	2x	2b	PBP 2x / PBP 2b
1	R6*			I_1	I_1	
2	RIE-919	0.12		I_1	I_1	
3	RIE-8442	0.12		I_2	I_1	
4	RIE-6083	0.25		I_1	I_1	
5	RIE-11080	0.25		II_2	I_2	
6	RIE-7184	0.25		I_3	I_1	
7	D-680	0.5		I_1	I_1	
8	RIE-2905	1		II_1	I_2	
9	WH-563	1		II_2	I_1	
10	RIE-11825		2	II_1	II_1	
11	RIE-12825		2	II_1	II_1	
12	BRI-249		2	II_2	II_1	
13	BRI-251		2	II_1	II_1	
14	NMH-285		2	II_1	II_1	
15	D-774		2	II_1	II_1	
16	RIE-13593		2	II_1	II_1	
17	RIE-13678		2	II_1	II_1	
18	WH-565		2	II_1	II_1	
19	NMH-158		8	II_1	II_1	



R6 non-capsulated-penicillin-susceptible *S. pneumoniae* control strain, **M** lambda DNA ladder marker

Table 4.4. Relationship of RFLP patterns, PFGE patterns, capsular serotypes and MICs of 18 penicillin-resistant *S. pneumoniae* clinical isolates from the UK. The results arranged according to decreasing susceptibility to penicillin

Strain	Penicillin MIC (mg/L)	Serotype	PFGE	PCR pattern	RFLP of PBP	
					2X	2B
RIE-919	0.12	6	D_1	III	I_1	I_1
RIE-8442	0.12	6	D_2	III	I_2	I_1
RIE-6083	0.25	29	H	III	I_1	I_1
RIE-11080	0.25	6	A_1	IV	II_2	I_2
RIE-7184	0.25	19	K	VI	I_3	I_1
D-680	0.5	9	L	VI	I_1	I_1
RIE-2905	1	19	F	II	II_1	I_2
WH-563	1	9	I	IV	II_2	I_1
RIE-11825	2	9	E	I	II_1	II_1
RIE-12825	2	9	C_1	I	II_1	II_1
BRI-249	2	6	C_2	I	II_2	II_1
BRI-251	2	9	A_1	I	II_1	II_1
NMH-285	2	9	A_2	I	II_1	II_1
D-774	2	9	A_3	I	II_1	II_1
RIE-13593	2	23	B_1	II	II_1	II_1
RIE-13678	2	23	G	II	II_1	II_1
WH-565	2	9	J	V	II_1	II_1
NMH-158	8	9	B_2	II	II_1	II_1

PFGE Pulsed-field gel electrophoresis, **RFLP** Restriction fragment length polymorphism, **PBP** Penicillin binding protein.

\+ altered pattern, - not altered pattern.

4.4. Discussion

The acquisition and spread of resistance in *S. pneumoniae* are complex processes, involving clonal spread, DNA horizontal transfer and point mutations. Molecular techniques have been used to analyse the nature of penicillin- and multiresistant pneumococci recovered in different countries. Thorough analysis of these isolates requires the use of techniques that index both their overall genetic relatedness and the relatedness of their altered *pbp2b, 2x* and *1a* genes (Dowson & Coffey, 1998). The variation in both the overall genotype and the three *pbp* genes allows the distinction between clonal spread of resistant pneumococci, and the generation of novel resistant clones by horizontal gene transfer, indicated by the presence of the same *pbp* gene(s) in strains that have different overall genotypes.

Several different approaches have been used for the molecular analysis of penicillin resistance in pneumococci. Jalal *et al.* (1997) used a PCR protocol to amplify susceptible genotypes of *pbp2b* and *2x* genes, and resistant genotype of *pbp1a* gene. Gillespie *et al.* (1997) used a gene fingerprinting or RFLP analysis of amplified *pbp2x, 2b* and *1a* genes but O'Neill *et al.* (1999) have used RFLP of amplified *pbp2b* only to detect penicillin susceptibility. PFGE macrorestriction pattern of chromosomal DNA was also used to evaluate the genetic relatedness of *S. pneumoniae* isolates (Hall *et al.*, 1996).

PFGE is a popular technique and has been widely used to compare genetically pneumococci isolated worldwide. Disadvantages of PFGE include the long and delicate procedure that is required for isolating and digesting high molecular weight genomic DNA, the need for expensive sophisticated equipment and the need for extended electrophoresis times. Restriction enzyme analyses of *pbp2b, 2x* and *1a* genes are often used to assist in the epidemiological analysis of pneumococci or as a method to detect penicillin susceptibility by pattern analysis. These methods prove to

give more precise information than serotyping and are very useful for the epidemiological investigation of penicillin resistance in pneumococci.

In the current study, the usefulness of PFGE macrorestriction with the *Sma*I enzyme of chromosomal DNA and DNA restriction patterns of amplified *pbp2b, 2x* and *1a* genes were determined. Eighteen penicillin-resistant strains were typed by PFGE. Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that only 2 of the isolates were identical. Taking a cut-off value of 80% similarity produced 4 clusters of isolates containing 4, 2, 2 and 2 strains respectively; the remaining 8 isolates were unrelated. Also isolates of related genotypes were found in a variety of geographic locations. It appears, therefore, that penicillin resistance was not simply the result of clonal spread but rather because of the presence of several genotypes. Our data also clearly showed that pneumococcal strains with the same serotype might show quite different PFGE patterns. This confirms that capsular serotyping is not a good criterion for genetic relatedness and PFGE is a more discriminative method.

Screening for changes in PBPs showed that there was a correlation between resistance and alteration in PBP. All 10 strains with an MIC of penicillin of 2 mg/L or above had changes in *pbp1a* and all, but 1, also had a change in *pbp2b*. In contrast only 6 had alterations in *pbp2x*. Isolates with MICs of penicillin less than 1 mg/L showed a more heterogeneous pattern of PBP changes and 2 isolates had no change in *pbp1a, 2x* or *2b*. This approach had difficulty in detecting resistant isolates, simply because the sequence of these strains are not recognised by primers used in PCR and gave false negative results.

To overcome this problem, restriction enzyme analysis of *pbp2b, 2x* and *1a* genes have been used. In this approach primers are designed to amplify the whole genome of *pbp2x, 2b* and *1a* and the PCR products were restricted by *Hin*fI endonuclease. *S. pneumoniae* strains with an MIC of penicillin of 1 mg/L or above exhibited

considerable homogeneity in *pbp2b* and *2x* RFLP. However, strains with an MIC less than 1 mg/L gave more heterogeneous patterns. The *pbp1a* gene failed to amplify.

These results suggest that penicillin resistance in *S. pneumoniae* in the UK is evolving in different strains and this is a cause for concern. Also it demonstrates the importance of using the available molecular techniques like PFGE and RFLP for early diagnosis of penicillin resistance.

CHAPTER FIVE

Activity of Quinolones against Penicillin Susceptible and Penicillin-Resistant Clinical Isolates of *S. pneumoniae*

5.1. Introduction

S. pneumoniae is an important pathogen and a major aetiologic agent of respiratory tract infections. Effective treatment of pneumococcal infections has until recently relied upon the use of β-lactam antibiotics, but with the emergence of antibiotic resistance, their use is now increasingly compromised (Baquero, 1995; Appelbaum, 1997). There is considerable interest in alternative antimicrobials, such as the fluoroquinolones. The quinolones developed over the past 10 to 15 years exhibit a broad spectrum of *in vitro* activity against a wide range of microorganisms (Wolfson & Hooper, 1989). Both the potencies and spectra of activity of the older quinolones have been increased with the introduction of newer agents.

Although presently available fluoroquinolones, such as ciprofloxacin and ofloxacin, are limited in their effectiveness against *S. pneumoniae* strains (Korner *et al.,* 1994; Bauernfeind, 1997), newer developmental compounds show greater promise (Barry *et al.,* 1996; Brueggemann *et al.,* 1997). Sparfloxacin has excellent activity against both penicillin-resistant and erythromycin-resistant strains of *S. pneumoniae* (Goa *et al.,* 1997). The two new quinolones moxifloxacin and trovafloxacin have similar activities and

are the most active of the current quinolones (Brueggemann *et al.,* 1997; Klugman & Gootz, 1997).

In this study I examined the activity of ciprofloxacin and four newer fluoroquinolones (Moxifloxacin, trovafloxacin, sparfloxacin and grepafloxacin) against 11 penicillin-susceptible and 18 genetically characterised penicillin-resistant *S. pneumoniae* isolates from the UK (see Chapter 4).

5.2. Materials & Methods

5.2.1. Bacterial Strains

Eleven penicillin-susceptible and 18 penicillin-resistant *S. pneumoniae* clinical isolates from the UK were examined for their susceptibility against five quinolones. Control strains included were: *S. aureus* NCTC 6571, *E. coli* NCTC 10418, *P. aeruginosa* NCTC 10662 and *S. pneumoniae* R6.

5.2.2. Antimicrobial Agents

Antimicrobial agents were supplied as sterile powders from different suppliers (Table 5.1). Drugs were stored as dry powders in darkness at 4°C. The antimicrobial solutions were freshly prepared with sterile MilliQ water when required.

Table 5.1. Antimicrobial Agents

Antimicrobial Agent	Supplier
Ciprofloxacin	Bayer plc
Moxifloxacin	Bayer plc
Grepafloxacin	Bayer plc
Trovafloxacin	Bayer plc
Sparfloxacin	Bayer plc

5.2.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed on Iso-Sensitest agar plates supplemented with 5% horse blood. MICs were determined by agar dilution of the antibacterial agents. Stock solutions of antibiotics were prepared immediately prior to use and two-fold dilutions of each antibiotic were prepared in agar plates. The media containing antimicrobial agents were used within 24 h.

Bacterial strains were grown overnight in Iso-Sensitest Broth (Oxoid, UK) at 37°C with continuous shaking. Plates containing antimicrobial agents were inoculated by transferring 1 μl of the undiluted overnight culture to the surface of the agar with a mulipoint inoculator (Denly, Billinghurst, UK) to give a final inoculum size of 10^4 CFU. A plate with no added antibiotic was inoculated as a positive control. All plates were incubated at 37°C for 18-24 h. The lowest concentration of the antimicrobial agents to inhibit all visible growth was determined as the MIC (mg/L).

5.3. Results & Discussion

S. pneumoniae no longer has predictable antibiotic susceptibility since isolates with increased resistance to penicillin and other antibiotics have become more common. There is a need for antimicrobial agents that can be used for oral and parental therapy of infections caused by penicillin-resistant pneumococci.

The current study examined the activity of ciprofloxacin and other new quinolones against a range of penicillin-susceptible and -resistant *S. pneumoniae* clinical isolates from centres throughout the UK. Table 5.2 shows the MICs results of ciprofloxacin, moxifloxacin, trovafloxacin, sparfloxacin and grepafloxacin.

Newer quinolones were the most active against these isolates. Ciprofloxacin had the lowest activity with MICs 4 to -16 fold higher than other quinolones. As observed with penicillin-susceptible pneumococci, all quinolones are generally 2 to 14-fold more active than against penicillin-resistant strains. Making the assumption that the breakpoint for the newer quinolones will be 2mg/L (it has not yet been decided), susceptibilities to newer quinolones were 100% for both penicillin-susceptible and -resistant isolates. Three penicillin-resistant isolates had high MICs of ciprofloxacin of $\geq$ 2 mg/L (strains D-680, RIE-11080 and BRI-251) and were selected for more investigation into the possible cause of elevated MIC to ciprofloxacin (see Chapter 6).

Table 5.3 shows the distribution of different quinolone MIC results over the PFGE patterns and capsular serotypes for the 18 penicillin-resistant isolates. Analysis of these results showed no correlation between particular genetic types and levels of quinolone susceptibility.

Table 5.2. MICs of 11 penicillin-susceptible and 18 penicillin-resistant *S. pneumoniae* strains to quinolones antimicrobial agents

Drug	MIC Range(mg/L)	MIC_{50}	MIC_{90}
Penicillin-susceptible			
Ciprofloxacin	0.008-0.5	0.5	0.5
Moxifloxacin	0.004-0.25	0.008	0.25
Trovafloxacin	0.004-0.25	0.004	0.25
Sparfloxacin	0.004-0.25	0.06	0.25
Grepafloxacin	0.004-0.12	0.004	0.12
Penicillin-resistant			
Ciprofloxacin	0.12-4	0.5	2
Moxifloxacin	0.016-0.5	0.12	0.25
Trovafloxacin	0.008-1	0.12	0.5
Sparfloxacin	0.03-1	0.12	0.25
Grepafloxacin	0.03-1	0.12	0.25

Table 5.3. Relationship between penicillin MICs, quinolone MICs, PFGE patterns and capsular serotypes of 18 penicillin-resistant *S. pneumoniae* clinical isolates from the UK

Starin	Serotype	PFGE	MIC (mg/L)					
			Pen	Cipr	Trov	Spar	Grep	Moxi
RIE-919	6	$D_{(1)}$	0.12	0.12	0.12	0.12	0.12	0.016
RIE-8442	6	$D_{(2)}$	0.12	0.25	0.008	0.25	0.25	0.03
RIE-6083	29	H	0.25	0.5	0.12	0.12	0.12	0.016
RIE-11080	6	$A_{(1)}$	0.25	2	0.5	0.25	0.25	0.25
RIE-7184	19	K	0.25	0.5	0.12	0.25	0.12	0.12
D-680	9	L	0.5	4	1	1	1	0.5
RIE-2905	19	F	1	0.5	0.008	0.03	0.12	0.06
WH-563	9	I	1	0.5	0.06	0.06	0.03	0.12
RIE-11825	9	E	2	0.25	0.008	0.03	0.25	0.03
RIE-12825	9	$C_{(1)}$	2	0.5	0.008	0.03	0.12	0.12
BRI-249	6	$C_{(2)}$	2	0.5	0.12	0.12	0.12	0.12
BRI-251	9	$A_{(1)}$	2	2	0.5	0.25	0.25	0.25
NMH-285	9	$A_{(2)}$	2	0.5	0.25	0.12	0.5	0.25
D-774	9	$A_{(3)}$	2	0.25	0.03	0.06	0.06	0.06
RIE-13593	23	$B_{(1)}$	2	0.5	0.06	0.12	0.25	0.12
RIE-13678	23	G	2	0.5	0.12	0.06	0.12	0.06
WH-565	9	J	2	1	0.25	0.25	0.25	0.12
NMH-158	9	$B_{(2)}$	8	0.5	0.06	0.06	0.12	0.12

MIC Minmum inhibitory concentration, **Pen** Penicillin, **Cipr** Ciprofloxacin, **Moxi** Moxifloxacin, **Trov** Trovafloxacin, **Spar** Sparfloxacin, **Grep** Grepafloxacin, **PFGE** Pulsed-field elelectrophoresis

Fuchs *et al.* (1997) recently reported that there was no correlation between quinolone susceptibility in *S. pneumoniae* and penicillin resistance. Similar observations were recently reported from the Alexander Project Collaborative Group (Goldstein *et al.*, 1996). No theoretical link has been proposed between penicillin and quinolone susceptibility, and the mechanisms of resistance to the two drugs are quite distinct. Contrasting data from the Northern Ireland Public Health Laboratory in Belfast are recently reported (Goldsmith *et al.*, 1998). The ciprofloxacin susceptibility of 42 penicillin-resistant pneumococci were compared to those of 122 penicillin-susceptible isolates, isolated since 1994. Only one of the 42 penicillin-resistant (2.4%) was susceptible to ciprofloxacin (MIC ≤1 mg/L) and 18 (42.9%) were fully resistant (MIC ≥4 mg/L). In comparison 84 out of 122 (68.8%) penicillin-susceptible were ciprofloxacin susceptible and only 7 (5.7%) were fully resistant. Thus ciprofloxacin resistance in pneumococci isolated in Belfast appears to be more common in penicillin-resistant strains than in penicillin-susceptible strains. It would appear, therefore, that there may be two phenomena occurring in Northern Ireland to explain the increased resistance to ciprofloxacin in local pneumococci generally and in penicillin-resistant strains in particular (Goldsmith *et al.*, 1998): the frequency of prescription of ciprofloxacin is higher than that in the rest of the UK or may be that ciprofloxacin-resistant pneumococci have spread clonally more quickly than ciprofloxacin-susceptible strains. However, strains have not yet been examined for possible clonal spread nor the activity of newer quinolone have been detected.

In summary, ciprofloxacin has been reported to be marginally active against pneumococcal (Fuchs *et al.*, 1997). Consequently, it should not be used as first-line treatment for pneumococcal infections. In contrast, the newer quinolones may be promising therapeutic options in patient populations that are likely to be infected with multiply resistant pneumococci (Brueggmann *et al.*, 1997).

CHAPTER SIX

Molecular Characterisation of Mutations in the QRDRs of *gyrA* and *parC* Genes in *S. pneumoniae* Clinical Isolates with High MICs to Ciprofloxacin

6.1. Introduction

A problem associated with the use of fluoroquinolones is the selection of spontaneous resistant mutants. Recent studies of *S. pneumoniae* have defined the appearance of ciprofloxacin-resistant pneumococci *in vitro* and *in vivo* (Tankovic *et al.,* 1996). Several studies with pneumococci have shown that low-level resistance (MIC 4-8 mg/L) can result from mutations in *parC* (Pan & Fisher, 1996; Muñoz & de la Campa, 1996; Janoir *et al.,* 1996), while increased levels of resistance (MIC 16-64 mg/L) occur following the acquisition of additional mutations in *gyrA* (Pan *et al.*, 1996; Muñoz & de la Campa, 1996; Janoir *et al.,* 1996). More recently a further resistance mechanism has been described in pneumococci involving the efflux of hydrophilic fluoroquinolones which shows a low-level of resistance (Brenwald *et al.,* 1997; Zeller *et al.,* 1997). Like the multi-drug efflux pumps NorA in *S. aureus* (Neyfakh *et al.,* 1993) and Bmr in *Bacillus subtilis* (Ahmed *et al.,* 1995), the efflux-mediated resistance described in pneumococci causes reduced susceptibility to several fluoroquinolones as well as variety of unrelated compounds (Brenwald *et al.,* 1997; Zeller *et al.,* 1997). Recently, Brenwald *et al.* (1998b) showed that efflux-mediated resistance in pneumococci is common amongst clinical isolates and involves a gene encoding a 399 amino-acid protein, which shows homology to NorA and Bmr. Reserpine was used as an inhibitor of the putative efflux pump of *S. pneumoniae* to

determine the presence or absence of this mechanism of resistance to fluoroquinolones (Brenwald *et al.*, 1997).

The location of *gyrA* and *parC* genes in the *S. pneumoniae* genome was revealed (Muñoz & de le Campa, 1996). Chromosomal DNA prepared from strain R6 was digested with different restriction endonucleases and was subjected to PFGE, and the resulting fragments were blotted and hybridised with probes specific for *parC, gyrA* and *gyrB*. The results indicate that *gyrA* is located in a region different from the region where *gyrB* and *parC* are located, according to the published *S. pneumoniae* R6 chromosomal map (Gasc *et al.*, 1991).

6.2. Materials & Methods

6.2.1. Bacterial Strains

The *S. pneumoniae* strains used in this study are part of the penicillin-resistant clinical isolates from the UK (see Chapter 5, Table 5.3). Three wild-type strains (RIE-11080, RIE-251 and D-680) with ciprofloxacin MIC of ≥2 mg/L were used. Also two wild-type ciprofloxacin-susceptible strains (R6 and RIE-919) were used for comparison.

6.2.2. Selection of Mutants

The wild-type ciprofloxacin-resistant D-680 strain was used for selection of ciprofloxacin-resistant mutants by stepwise selection. First-step mutants of *S. pneumoniae* D-680 were selected by incubating 10^8 CFU onto Columbia agar (Oxoid, UK) supplemented with 5% horse blood and containing ciprofloxacin (Brenwald *et al.*, 1998). Following incubation at 37°C in an atmosphere of 5-10% CO_2 for 3 days, mutants were subcultured onto ciprofloxacin-free medium.

6.2.3. MIC Determination for Ciprofloxacin and Norfloxacin in the Presence or Absence of Reserpine

Reserpine was used as an inhibitor of the putative efflux pump of *S. pneumoniae* to determine the presence or absence of efflux pump mechanism of resistance to fluoroquinolones. The susceptibility to ciprofloxacin (Bayer,UK) and norfloxacin (Sigma, UK) in the presence or absence of 20 μg/ml of reserpine (Sigma, UK) were determined by an agar dilution method with Iso-Sensitest agar (Oxoid, UK) supplemented with 5% horse blood with an inoculum of 10^4 CFU/spot (Brenwald *et al.*, 1998a). Reserpine was freshly prepared before use, and media containing it were

used immediately. After incubation at 37°C in 5-10% CO_2 for 18 h, the MIC was recorded as the lowest antibiotic concentration inhibiting growth.

6.2.4. Isolation of Chromosomal DNA

S. pneumoniae strains was cultivated on Columbia agar (Oxoid, UK) supplemented with 5% horse blood and incubated in 5-10% CO_2 at 37°C overnight. Colonies were emulsified in 50 µl sterile MilliQ water in a microcentrifuge tube and boiled for 5 min; after centrifugation the supernatant contained the DNA which was used for PCRs.

6.2.5. DNA Amplification by Polymerase Chain Reaction

The PCR protocol used to amplify the QRDRs of *gyrA* and *parC* genes was a modification of that used by Pan *et al* (1996). The primers used (Table 6.1) were supplied high-pressure liquid chromatography (HPLC) purified by Oswel DNA Services (Southhampton, UK).

Table 6.1. Primers sequence used in PCR to amplify the QRDRs of *gyrA* & *parC* in *S. pneumoniae*

Name	Sequence (5`__3`)
gyrA	CCGTCGCATTCTCTACG
gyrA`	AGTTGCTCCATTAACCA
parC	TGGGTTGAAGCCGGTTCA
parC`	TGCTGGCAAGACCGTTGG

All the PCR reagents were supplied by Advanced Biotechnologies Ltd., UK. The optimal reaction mix included 0.5 µl of *Taq* DNA Polymerase (5 U/µl), 10 µl of 10x Reaction buffer [200 mM $(NH_4)_2SO_4$, 750 Mm Tris-HCl (pH 8.8), 0.1% Tween®20], 5 µl dNTP Mix (20 mM), 3 µl $MgCl_2$ (25 mM), 1 µl primers Mix (10 pmole/µl), 5 µl of DNA template, and sterilised MilliQ water to a final volume of 100 µl. This was overlaid with sterile mineral oil and processed on Techne Thermal Cycler (Techne Cambridge Ltd., UK). The optimal PCR cycling conditions were as follows: 1 cycle of 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min followed by a final cycle of 72°C for 7 min. A negative control was included in each PCR run where DNA was replaced by an equal amount of sterilised MiliQ water. PCR products were analysed by electrophoresis through a 1.8 % agarose-ethidium bromide-containing gel with 100 bp DNA ladder (GibcoBRL Life technologies) as marker and bands visualised with UV transillumination.

6.2.6. Restriction Fragments Length Polymorphism with *Hin*fI

Restriction of the *gyrA* and *parC* PCR products was performed using *Hin*fI (Promega, UK). The digestion mixture consisted of 1 µl of *Hin*fI enzyme (10 U/µl) in 10 mM Tris-HCl (pH 7.5), 60 mM $NaCl_2$, 7 mM $MgCl_2$, 0.1 mg bovine serum albumin per liter in 50 µl volume. A total of 20 µl of PCR product was added to 2 µl of buffer and 1 µl of *Hin*fI enzyme. Digestion proceeded for 4 h at 37°C in a waterbath, and the products were run on a 1.8% agarose gel as described above.

6.2.7. DNA Sequencing

To identify mutations at the nucleotide sequence level, *gyrA* and *parC* PCR products were sequenced and analysed in an automatic DNA sequencer. Purified DNA samples were processed and analysed in the Department of Haematology in the Royal Infirmary of Edinburgh.

6.2.8. Pulsed-field Gel Electrophoresis (PFGE)

Chromosomal DNA embedded in agarose plugs was prepared from *S. pneumoniae* R6, D-680 wild-type and first-step mutant strains as described previously (see Chapter 4, section 4.2.3). PFGE was carried out after microrestriction of the chromosomal DNA with *Sma*I endonuclease.

6.3. Results & Discussion

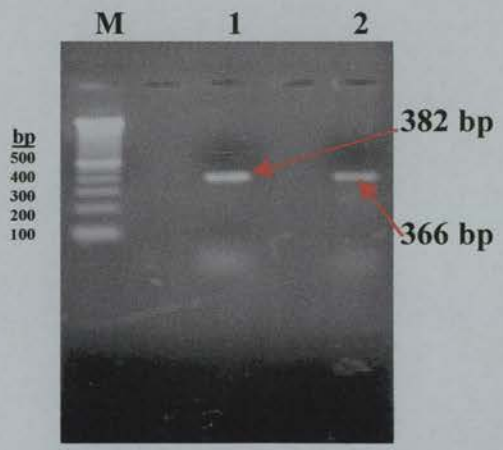
6.3.1. MIC Determination in the Presence of Reserpine

Reserpine had no effect on ciprofloxacin nor norfloxacin activity against the *S. pneumoniae* strains tested (Table 6.2). This suggests that no efflux pump mechanism was present in these strains.

6.3.2. Amplification of QRDR in *gyrA* and *parC* by PCR

Chromosomal DNA from *S. pneumoniae* strains was used as a template for PCR to amplify the QRDR of the *gyrA* and *parC* genes. Oligonucleotide primer pairs allowed amplification and isolation of 382 bp *gyrA* products (encoding residues 46 to 172) and 366 bp *parC* products (encoding residues 36 to 157), respectively)(Figure 6.1).

Figure 6.1. Agarose gel electrophoresis of GyrA and ParC PCR products from *S. pneumoniae* 680 wild-type strain



Lane M	100 bp DNA ladder marker
Lane 1	GyrA PCR product (382 bp)
Lane 2	ParC PCR product (366 bp)

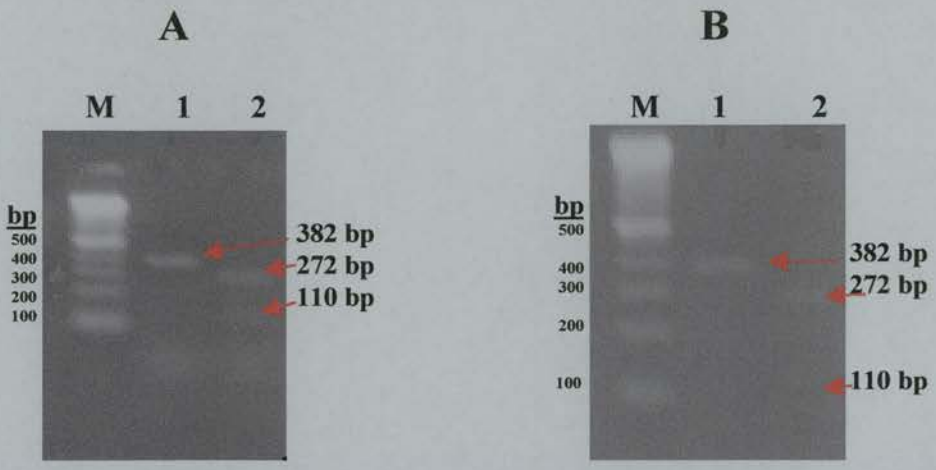
6.3.3. RFLP by *Hin*fI of *gyrA* and *parC* PCR Products

Initially, the status of the *gyrA* and *parC* QRDRs was examined by digesting PCR products with *Hin*fI and examining the resulting DNA fragments by electrophoresis. The 382 bp *gyrA* PCR product from all strains underwent cleavage at a single *Hin*fI site (overlapping the coding sequence for the conserved Ser codon which is equivalent to the resistance hot spot Ser-83 in *E. coli*), generating 110 and 272 bp fragments (Figure 6.2). The *gyrA* PCR product from the first-step mutant of strain D-680 was also cleaved by *Hin*fI, indicating that there is no mutation at either codon 82 nor 83.

The 366 bp *parC* product from the wild-type gene has *Hin*fI sites at nucleotide position 232 and 288 and on digestion with *Hin*fI generated 183, 127 and 56 bp fragments. Acquisition of a quinolone resistance mutation altering mutational hot spot at Ser-79 in ParC leads to a loss of the position 232 *Hin*fI site and generation of two 183 bp fragments. The *parC* genes of *S. pneumoniae* strains in each case retained all their *Hin*fI sites, which is consistent with the absence of Ser-79 codon changes (Figure 6.3). However, analysis by *parC Hin*fI RFLP of the first-step mutant of D-680 revealed that it gave a single 183 bp band suggesting acquisition of a Ser-79 change (Figure 6.3).

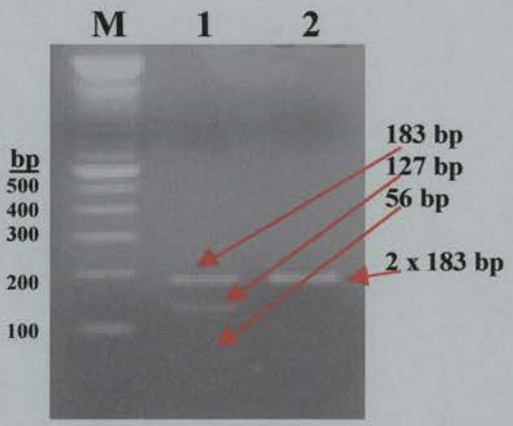
The RFLP analysis provided evidence for *parC* mutation in the first-step mutant and the absence of *gyrA* mutation. However, these observations did not exclude the presence of mutations at other positions in the QRDR of *gyrA* and *parC* genes.

Figure 6.2. A & B. Agarose gel electrophoresis of *Hin*fI restricted GyrA PCR product from *S. pneumoniae* D-680 wild-type strain and first-step mutant



A.	Lane M	100 bp DNA ladder marker
	Lane 1	GyrA PCR product from D-680 wild-type strain
	Lane 2	*Hin*fI restricted GyrA PCR product from D-680 wild-type strain
B.	Lane M	100 bp DNA ladder marker
	Lane 1	GyrA PCR product from D-680 first-step mutant
	Lane 2	*Hin*fI restricted GyrA PCR product from D-680 first-step mutant

Figure 6.3. Agarose gel electrophoresis of *Hin*fI restricted ParC PCR product from *S. pneumoniae* D-680 wild-type strain and first-step mutant



Lane M 100 bp DNA ladder marker

Lane 1 *Hin*fI restricted ParC PCR product from D-680 wild-type strain

Lane 2 *Hin*fI restricted ParC PCR product from D-680 first-step mutant

6.3.4. Sequencing of the QRDR in *gyrA* and *parC*

To analyse the respective QRDRs at the nucleotide level, PCR products from all *S. pneumoniae* strains and the first-step mutant were subjected to automated DNA sequencing (Figures 6.4 & 6.5). Consistent with the RFLP data, all wild-type strains showed no mutations in the *Hin*fI restriction site in *gyrA* and *parC* QRDRs.

However the first-step mutant derived from D-680 strain had acquired mutations in *gyrA* and *parC* QRDRs at Glu-87 (GAA)-to-**Lys** (AAA) and at Ser-79 (TCT)-to-**Tyr** (TAT), respectively. These genetic changes are consistent with the *parC* RFLP results, but not with *gyrA* results, and are associated with 8-fold increases in ciprofloxacin MIC in the first-step mutant compared with that for the wild-type D-680 strain. Interestingly, the cross-resistance profile of ciprofloxacin-selected mutants against the newer quinolones (Table 6.2) showed that the single mutations selected by stepwise drug challenge are identical to those at the equivalent positions of *gyrA* and *parC* mutations which are known to cause quinolone resistance in other species.

6.3.4.1. Results of *gyrA* QRDR sequence of *S. pneumoniae*

Figure 6.4. A, B & C

A partial 382-base region of partial *gyrA* gene was sequenced and amino acid residues at position 46 to 172 including QRDR are shown below. The *Hin*fI restriction site are grey boxed. Letters under the nucleotide sequence show the deduced protein sequence. Amino acid residues are numbered under the letters by analogy with *E. coli* GyrA protein. Red letters in the sequence indicate nucleotides and amino acids different from that of *S. pneumoniae* ciprofloxacin-susceptible R6 strain and only the section where nucleotide changes occur is shown.

Figure 6.4.A. *gyrA* QRDR sequence of *S. pneumoniae* ciprofloxacin-susceptible R6 strain, as listed in GENBANK (AF053121), with MIC of 0.5 mg/L (Balas *et al.*, 1998).

```
261 c cgt cgc att ctc tat gga atg aat gaa ttg ggt gtg
       R   R   I   L   Y   G   M   N   E   L   G   V
      46

298   acc cca gac aaa ccc cat aaa aaa tct gct cgt att
       T   P   D   K   P   H   K   K   S   A   R   I

334   aca ggg gat gtc atg ggt aaa tac cac cca cac ggg
       T   G   D   V   M   G   K   Y   H   P   H   G

370   gat tcc tct att tat gaa gcc atg gtc cgt atg gct
       D   S   S   I   Y   E   A   M   V   R   M   A
           83                  87
406   caa tgg tgg agc tac cgt tac atg ctt gta gat ggt
       Q   W   W   S   Y   R   Y   M   L   V   D   G

442   cat ggg aat ttt ggt tcc atg gat gga gat agt gct
       H   G   N   F   G   S   M   D   G   D   S   A

478   gcc gct caa cgt tat acc gag gca cgt atg agc aag
       A   A   Q   R   Y   T   E   A   R   M   S   K

514   att gct ctg gaa atg ctt cgt gat atc aac aaa aat
       I   A   L   E   M   L   R   D   I   N   K   N

550   aca gtt gat ttc gtt gat aac tat gat gcc aat gaa
       T   V   D   F   V   D   N   Y   D   A   N   E

586   cgg gaa ccc ttg gtc ttg cca gcg cgt ttt cca aac
       R   E   P   L   V   L   P   A   R   F   P   N

622   ctt ttg gtt aat gga gca act 642
       L   L   V   N   G   A   T
                           172
```

Single-letter amino acid codes

A alanine, **C** cysteine, **D** aspartic acid, **E** glutamic acid, **F** phenylalanine, **G** glycine, **H** histidine, **I** isoleucine, **K** lysine, **L** leucine, **M** methionine, **N** aspargine, **P** proline, **Q** glutamine, **R** arginine, **S** serine, **T** threonine, **V** valine, **W** tryptophan, **Y** tyrosine.

Figure 6.4.B. Four *gyrA* QRDR sequences of *S. pneumoniae* strain RIE-919 (MIC 0.12 mg/L), RIE-11080, BRI-251 and D-680 (MIC 2, 2 and 4 mg/L, respectively)

-All together only one nucleotide was altered resulting in no change of the amino acid. This altered nucleotide is also present at this sequence if it is compared with *gyrA* ORDR sequence of *S. pneumoniae* ATCC 49619 (GENBANK AF065152) or *S. pneumoniae* NCTC 7465 (GENBABK SPU37560).

```
334  aca ggg gat gtc atg ggt aaa tat cac cca cac ggg
      T   G   D   V   M   G   K   Y   H   P   H   G

370  gat tcc tct att tat gaa gcc atg gtc cgt atg gct
      D   S   S   I   Y   E   A   M   V   R   M   A
          83                  87

406  caa tgg tgg agc tac cgt tac atg ctt gta gat ggt
      Q   W   W   S   Y   R   Y   M   L   V   D   G
```

Figure 6.4.C. *gyrA* QRDR sequence of ciprofloxacin-resistant *S. pneumoniae* first-step mutant of strain D-680 with an MIC of 32 mg/L

-Two nucleotides within QRDR were altered resulting in 1 amino acid mutation: **Glu-87-Lys**.

```
334  aca ggg gat gtc atg ggt aaa tat cac cca cac ggg
      T   G   D   V   M   G   K   Y   H   P   H   G

370  gat tcc tct att tat aaa gcc atg gtc cgt atg gct
      D   S   S   I   Y   K   A   M   V   R   M   A
          83                87

406  caa tgg tgg agc tac cgt tac atg ctt gta gat ggt
      Q   W   W   S   Y   R   Y   M   L   V   D   G
```

6.3.4.2. Results of *parC* QRDR sequence of *S. pneumoniae*

Figure 6.5. A, B & C

A partial 366-base region of partial *parC* gene was sequenced and amino acid residues at position 36 to 157 including QRDR are shown below. The *Hin*fI restriction site are grey boxed. Letters under the nucleotide sequence show the deduced protein sequence. Nucleotides and amino acids are numbered by taking the first *parC* nucleotide as nucleotide 1 and the first ParC residue as number 1. Red letters in the sequence indicate nucleotides and amino acids different from that of *S . pneumoniae* ciprofloxacin-susceptible R6 strain and only section where nucleotide changes occur is shown.

Figure 6.5.A. *parC* QRDR sequence of S. *pneumoniae* ciprofloxacin-susceptible R6 strain, as listed in GENBANK (X95717), with MIC of 0.5 mg/L (Muñoz & de la Campa, 1996)

```
105  t ggg ttg aag ccg gtt caa cgc cgt att ctt tat
       G   L   K   P   V   Q   R   R   I   L   Y
      36

139    tct atg aat aag gat agc aat act ttt gac aag
        S   M   N   K   D   S   N   T   F   D   K

172    agc tac cgt aag tcg gcc aag tca gtc ggg aac
        S   Y   R   K   S   A   K   S   V   G   N

205    atc atg ggg aat ttc cac cca cac ggg gat tct
        I   M   G   N   F   H   P   H   G   D   S
                                                79

238    tct atc tat gat gcc atg gtt cgt atg tca cag
        S   I   Y   D   A   M   V   R   M   S   Q
                    83

271    aac tgg aaa aat cgt gag att cta gtt gaa atg
        N   W   K   N   R   E   I   L   V   E   M

304    cac ggt aat aac ggt tct atg gac gga gat cct
        H   G   N   N   G   S   M   D   G   D   P

337    cct gcg gct atg cgt tat act gag gca cgt ttg
        P   A   A   M   R   Y   T   E   A   R   L

370    tct gaa att gca ggc tac ctt ctt cag gat atc
        S   E   I   A   G   Y   L   L   Q   D   I

403    gag aaa aag aca gtt cct ttt gca tgg aac ttt
        E   K   K   T   V   P   F   A   W   N   F

436    gac gat acg gag aaa gaa cca acg gtc ttg cca
        D   D   T   E   K   E   P   T   V   L   P

469    gca 471
        A
       157
```

Figure 6.5.B. Four *parC* QRDR sequences of *S. pneumoniae* strain RIE-919 (MIC 0.12 mg/L), RIE-11080, BRI-251 and D-680 (MIC 2, 2 and 4 mg/L, respectively)

-All together only one nucleotide was altered resulting in no change of the amino acid.

```
172    agc tac cgt aag tcg gcc aag tca gtc ggg aac
        S   Y   R   K   S   A   K   S   V   G   N

205    atc atg ggg aat ttc cac cca cac ggg gat tct
        I   M   G   N   F   H   P   H   G   D   S
                                                79

238    tct atc tat gac gcc atg gtt cgt atg tca cag
        S   I   Y   D   A   M   V   R   M   S   Q
                    83

271    aac tgg aaa aat cgt gag att cta gtt gaa atg
        N   W   K   N   R   E   I   L   V   E   M
```

Figure 6.5.C. *parC* QRDR sequence of ciprofloxacin-resistant *S. pneumoniae* first-step mutant of strain D-680 with an MIC of 32 mg/L

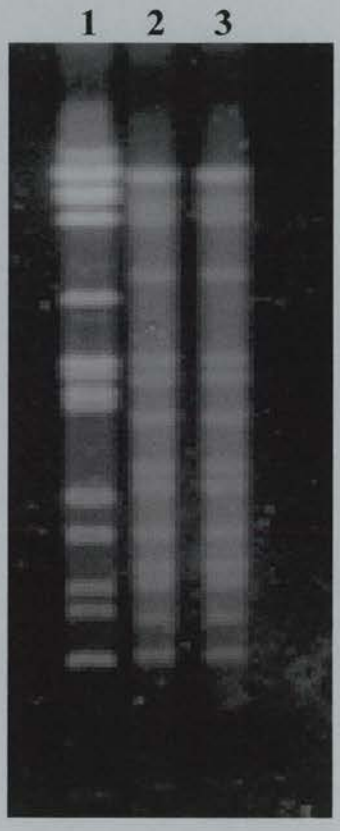
-Two nucleotide within QRDR was altered resulting in 1 amino acid mutation: **Ser-79-Tyr**. This led to the loss of the position 232 *Hin*fI restriction site.

```
172    agc tac cgt aag tcg gcc aag tca gtc ggg aac
        S   Y   R   K   S   A   K   S   V   G   N

205   atc atg ggg aat ttc cac cca cac ggg gat tat
       I   M   G   N   F   H   P   H   G   D   Y
                                                79

238    tct atc tat gac gcc atg gtt cgt atg tca cag
        S   I   Y   D   A   M   V   R   M   S   Q
                    83

271    aac tgg aaa aat cgt gag att cta gtt gaa atg
        N   W   K   N   R   E   I   L   V   E   M
```

6.3.5. PFGE Results

The comparison of patterns of fragments generated by PFGE of chromosomal DNA from D-680 wild-type and first-step mutant strains showed identical restriction patterns (Figure 6.6). This suggested that no point mutation or DNA rearrangement had occured in the first-step mutant during the selection step by ciprofloxacin from the wild-type strain.

Figure 6.6. PFGE of *Sma*I digested genomic DNA from *S. pneumoniae* strains



Lane 1 R6 strain
Lane 2 D-680 wild-type strain
Lane 3 D-680 first-step mutant

Table 6.2. Identification of GyrA and ParC mutations in wild-type strains and ciprofloxacin-selected mutants

Strain	Mutation		Minimum Inhibitory Concentration (mg/L)							
	gyrA	*parC*	Cip	Cip + R	Nor	Nor + R	Spar	Grep	Trov	Moxi
R6	—	—	0.5	0.5	8	8	0.12	0.06	0.12	0.03
RIE-919	None	None	0.12	0.12	8	8	0.12	0.12	0.12	0.016
BRI-251	None	None	2	2	64	64	0.25	0.25	0.5	0.25
RIE-11080	None	None	2	2	64	64	0.25	0.25	0.5	0.25
D-680 wild-type	None	None	4	4	64	64	1	1	1	0.5
D-680 first-step mutant	**Glu-87-Lys**	**Ser-79-Tyr**	32	32	128	128	4	16	16	8

Cip ciprofloxacin, **Nor** norfloxacin, **R** Reserpine, **Moxi** moxifloxacin, **Trov** trovafloxacin, **Spar** sparfloxacin, **Cip** ciprofloxacin, **Grep** grepafloxacin.

CHAPTER SEVEN

Molecular Typing and β-Lactamase Analysis of Amoxycillin-Resistant Clinical Isolates of *H. influenzae*

7.1. Introduction

H. influenzae is one of the most common pathogens responsible for respiratory tract infections. β-lactams are widely used to treat such infections. Resistance to ampicillin was first documented in Europe in 1972 (Mathies, 1972). Since then, ampicillin resistance has risen steadily to the present level of 10-30% reported by most centres (Machka *et al.*, 1988).

The major mechanism of resistance to β-lactams drugs in *H. influenzae* is the production of β-lactamases, and the prevalence of β-lactamase-producing isolates has increased over the last 20 years (Powell *et al.*, 1987 & 1992; Barry *et al.*, 1994). The extent of non-β-lactamase-mediated resistance has always been difficult to assess and it is possible that β-lactamase production may be missed by routine testing (Scriver *et al.*, 1994). β-lactam resistance in strains apparently negative for β-lactamase production has been attributed to changes in penicillin binding proteins or alterations in permeability (Reid *et al.*, 1987).

The most common β-lactamase in *H. influenzae* is TEM-1, which accounts for more than 80% of β-lactamase-positive isolates (Scriver *et al.*, 1994). This accounts for the present susceptibility of these organisms to β-lactam/β-lactamase inhibitor combinations and

cephalosporins. A second β-lactamase, ROB-1, has also been detected in *H. influenzae* (Rubin *et al.,* 1981). Although less numerous than TEM-1, ROB-1 has been found in up to 8% of isolates in some studies (Daum *et al.,* 1988; Scriver *et al.,* 1994). A third β-lactamase, VAT-1, has also been identified recently in *H. influenzae* (Vali *et al.,* 1994). The significant of this enzyme is not clear at present, but it has been detected in isolates from different geographical centres in Scotland (Shanahan *et al.,* 1996).

β-Lactamases in *H. influenzae* confer resistance to β-lactam antibiotics. It is, therefore, essential that one can identify the production of β-lactamases by clinical isolates and have effective ways of distinguishing the different enzymes. This is necessary for epidemiological surveys, predicting future resistance trends and to ensure that patients receive the appropriate β-lactam or alternative therapy. The first indication of the presence of β-lactamases is usually the observation of increased levels of resistance in clinical isolates as detected by routine susceptibility testing. Analytical isoelectric focusing (IEF) can then be used to characterise the β-lactamase produced. Molecular techniques have become very effective for identifying different β-lactamases (Payne & Thomson, 1998). PCR has been used to detect ampicillin resistance genes in CSF samples containing *H. influenzae* (Tenover *et al.,* 1994). Correlation was obtained between the result of MIC testing, β-lactamase production as determined by nitrocephin and PCR testing (Tenover *et al.,* 1994). Molecular typing by pulsed-field gel electrophoresis has also been used to assess the clonal relationship of isolates (Gazagne *et al.,* 1998).

7.2. Materials & Methods

7.2.1. Bacterial Strains

Between 1995 and 1996, 231 isolates of *H. influenzae* were obtained from centres throughout the UK (see section 2.1). Twenty percent (46) of isolates were amoxycillin-resistant and, of these, 19% (9) were also resistant to co-amoxiclav. In this study, these isolates were further examined for possible mechanisms of resistance to amoxycillin and co-amoxiclav and genotyped by pulsed-field gel electrophoresis.

7.2.2. β-Lactamase Preparation

Test strains were inoculated onto the surface of Chocolate Blood Agar slopes and incubated at 37°C in a 5% CO_2 incubator overnight. Bacteria were washed off the surface of the agar with 1 ml of 50mM Sodium Phosphate buffer (pH 7.0) and transferred to an ice cold container. The cells were cooled on ice and disrupted by sonication (MSE Soniprep 150, MSE Instruments, Crawley, Sussex) at 4 x 15 sec pulses of 8μm amplitude separated by a 15 sec cooling period. The cell lysate was cleared by centrifugation at 4°C in an MSE Microcentaur centrifuge at 11,600g (13,000 rpm) for 20 min. The cell free supernatant was stored at -20°C until required.

7.2.3. Detection of β-Lactamase Production (Nitrocephin Spot Test)

The reaction time for a 30 µl volume of the β-lactamase preparation to change the colour of 100µl of chromogenic cephalosporins, nitrocephin, solution (50 mg/L) from yellow to red was taken as an indication of the β-lactamase activity of the enzyme preparation (O'Callaghan *et al.*, 1972).

7.2.4. Analytical Isoelectric Focusing

β-lactamases were identified by analytical isoelectric focusing (IEF) as described by Matthew *et al.*, (1975). β-lactamase extracts were focused on a thin layer polyacrylamide gel containing broad range ampholines (pH 3.5-10). The composition of the gel mixture is shown in Table 7.1.

Table 7.1. The composition of the Isoelectric focusing gel

Material	Supplier	Volume (ml)	Final Concentration
5% v/v Tetramethylethylenediamine (TEMED) in distilled water	**Sigma**	**0.2**	**0.25 mg/L**
40% w/v Ampholines pH 3.5-10	**Sigma**	**2**	**2% w/v**
Acrylamide (100g) & Methyl *bis*acrylamide (2.7 g) in 300 ml distilled water	**BDH**	**9**	**Acrylamide 76 g/L, *bis*acrylamide 2 g/L**
Distilled water		**25**	
Riboflavin (20 mg/L)	**Sigma**	**4**	**2 mg/L**

The polyacrylamide gel solution was poured between two glass plates of different sizes, the smaller of which was coated to promote the adhesion of the gel. The plate coating solution consisted of 0.5% w/v gelatin and 0.5% w/v chromium potassium sulphate (both Aldrich Chemical Co. Ltd, Dorset) in sterile distilled water, and was allowed to dry on the plate. The other glass was siliconised with Sigmacote siliconising solution to reduce adhesion. When poured, the acrylamide was polymerised by the riboflavin in the presence of ultraviolet light for 6 h.

When the glass plates were separated apart, the gel (which had adhered to the smaller plate) was allowed to dry in air for up to 1 h before loading with samples of β-lactamase preparation on to the gel surface close to the anode. The nitrocephin spot test time determined the volume of the β-lactamase preparation loaded onto the gel, the volume being calculated as the nitrocephin spot test time (see section 7.2.3). Up to a maximum of 50 μl of the β-lactamase preparation was loaded to any one lane.

Isoelectric focusing was carried out at a constant power of 1W, with the maximum settings of 500V and 20 mA overnight. The gel was calibrated by focusing β-lactamase of known pI. The focused β-lactamase bands were visualised by overlaying the surface of the gel with sheets of filter paper (Whatman No. 54, BDH), previously soaked in nitrocephin solution (500 mg/L). The bands of β-lactamase activity appeared red on a yellow background. Photographs of focused β-lactamases were taken with a Polaroid camera (setting B4, F8) with a Wratten 58 green filter.

7.2.5. Isolation of Chromosomal DNA

H. influenzae strains were cultivated on Chocolate Blood agar and incubated in 5-10% CO_2 at 37°C overnight. Colonies were emulsified in 50 µl sterile MiliQ water in a microcentrifuge tube and boiled for 3 min; supernatant containing DNA was used for PCRs.

7.2.6. DNA Amplification by Polymerase Chain Reaction

The PCR protocol used to amplify the bla_{TEM-1} gene in *H. influenzae* was a modification of that used by Tenover *et al.* (1994). The bla_{TEM-1} universal primers used (Table 7.2) were supplied after high-pressure liquid chromatography (HPLC) purification by Oswel DNA Services (Southhampton, UK).

Table 7.2. Primers sequence used in PCR to amplify the bla_{TEM-1} in *H. influenzae*

Name	Sequence (5`___3`)
bla_{TEM-1}	TGGGTGCACGAGTGGGTTAC
bla_{TEM-1}`	TTATCCGCCTCCATCCAGTC

All the PCR reagents were supplied by Advanced Biotechnologies Ltd., UK. The optimal reaction mix included 0.5 µl of *Taq* DNA Polymerase (5 U/µl), 10 µl of 10x Reaction buffer [200 mM $(NH_4)_2SO_4$, 750 Mm Tris-HCl (pH 8.8), 0.1% Tween®20], 5 µl dNTP Mix (20 mM), 3 µl $MgCl_2$ (25 mM), 1 µl primers Mix (10 pmole/µl), 5 µl of DNA template, and sterilised MiliQ water to a final volume of 100 µl. This was overlaid with sterile mineral oil and processed on Techne Thermal Cycler (Techne Cambridge Ltd., UK). The optimal PCR cycling conditions were as follows: 1 cycle of 94°C for 5 min

followed by 30 cycles of 94°C for 2 min, 57°C for 1 min and 72°C for 2 min followed by a final cycle of 72°C for 10 min. A negative control was included in each PCR run where DNA was replaced by an equal amount of sterilised MiliQ water. Also a β-lactamase-negative and a TEM-1 producing *H. influenzae* isolates were included as controls. PCR products were analysed by electrophoresis through a 1.8 % agarose-ethidium bromide-containing gel with 100 bp DNA ladder (GibcoBRL Life technologies) as marker and bands visualised with UV transillumination.

7.2.7. Pulsed-Field Gel Electrophoresis (PFGE)

Preparation of Intact Chromosomal DNA in Agarose Plugs

The preparation of intact chromosomal DNA in agarose plugs was compiled with some modifications from the method of Curran *et al.* (1994). *H. influenzae* strains were inoculated in Brain Heart Infusion broth (Oxoid, UK) supplemented with 10 μg/ml Hemin and 2 μg/ml Nicotinamide adenine dinucleotide (Sigma, UK) and were grown to a density of 2 x 10^9 cells/ml (optical density of 0.15 at 650 nm) in 37°C incubator with 5-10% CO_2 overnight. The cells were centrifuged at 4°C at 3,000 rpm in a centrifuge for 10 min. The cell pellets were resuspeneded in 0.5 ml of SB buffer [10 mM Tris-HCl (pH 7.5), 1 M NaCl], recentrifuged, resuspeneded finally in fresh SB buffer and held at 37°C. Each 0.5 ml cell suspension was then mixed with 0.5 ml of molten 2% w/v low-melt preparative grade agarose (Bio-Rad, UK) in distilled water. The mixture was micropipetted immediately into the wells of a perspex plug mould (Bio-Rad, UK) and cooled to 4°C to allow the agarose to set. Each set of plugs was then ejected from the mould into bijou bottles containing 2 ml of LB buffer [10 mM Tris-HCl (pH 7.5), 1 M NaCl, 100 mM EDTA, 0.5% polyxythylene 20 cetyl ether, 0.2% sodium deoxycholate, 0.5% *n*-lauroylsarcosine, 1 mg/ml lysozyme, and 20 μg/ml ribonuclease A] and the

bottles were incubated overnight at 37°C. The LB buffer was then replaced with 2 ml of PB buffer [0.5 M EDTA (pH 9.0), 1% *n*-lauroylsarcosine, 1 mg/ml proteinase K] and incubated at 55°C for 48 h. At the end of this period, the PB buffer was replaced with 2 ml of PMSF buffer [1 mM phenylmethylsulphonyl fluoride (PMSF), 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA] and incubated at room temperature for 2 h. This wash step was repeated once with fresh PMSF buffer, followed by three washes for a minimum of 2 h each in TE buffer [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA]. The plugs were then stored at 4°C in TE buffer until required.

Digestion of Chromosomal DNA in Agarose Plugs

Generally, digests were performed with DNA contained in about one-third of a complete plug. Each plug portion was transferred to a sterile microcentrifuge tube and equilibrated at 4°C in 100 µl of the *Sma*I digestion buffer (Promega, UK). After 1 h, the buffer was replaced with 100 µl of fresh digestion buffer and 12 U of *Sma*I (Promega, UK) was added. After 4-6 h digestion at 37°C, the buffer-enzyme mixture was replaced with 100 µl of inactivation buffer [0.5 M EDTA (pH 9.0), 1% *n*-lauroylsarcosine] and kept at 4°C until ready for electrophoresis.

Electrophoresis

DNA macrorestriction fragments were separated on 1% agarose gel by PFGE employing a CHEF-DR II system (Bio-Rad, UK) at 6 V/cm for 30 h with a switching time of 1-26 sec at 12°C. The gels were stained with 1 µg/ml of ethidium bromide (Sigma, UK) and were destained in water for 1h before photography.

Gelcompar Software

The PFGE patterns were analysed employing Gelcompar software (Applied Math, Kortrijk, Belgium). The PFGE patterns were compared by the UPGMA (unweighted pair group method with arithmetic averages) clustering method by employing the Dice coefficient (Shi *et al.*, 1996). A tolerance in the band position of 1.2% was applied during the comparison of PFGE fingerprinting patterns.

7.3. Results

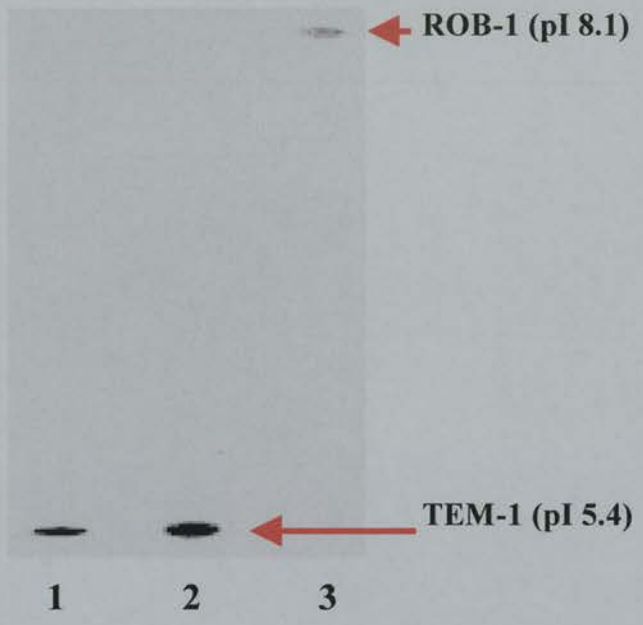
7.3.1. The Prevalence of β-Lactamase Production in *H. influenzae*

The cell-free extracts of the 231 isolates were prepared (section 7.2.2) and the β-lactamase activity of each preparation was assayed by the nitrocephin spot test. The change in colour of nitrocephin solution from yellow to red after 30 min was taken as an indication of β-lactamase activity. Based on this method 16% (37) of the *H. influenzae* isolates harboured a detectable β-lactamase enzyme (Table 7.3).

7.3.2. Characterisation of β-Lactamases in *H. influenzae*

To identify the β-lactamases in *H. influenzae*, sonicated extracts of the 37 β-lactamase producing isolates were examined by analytical isoelectric focusing. It was demonstrated that all the β-lactamase producing strains produced a single β-lactamase that was focused as a single band at pI 5.4 in IEF gel and co-focused with TEM-1β-lactamase control (Figure 7.1).

Figure 7.1. IEF pattern of the β-lactamases isolated from 37 β-lactamase producing *H. influenzae* isolates on 10% polyacrylamide gel containing broad-range ampholines (pH 3.5-10)



Lane 1	TEM-1 (pI 5.4) isolated from the β-lactamase producing *Haemophilus influenzae* isolates
Lane 2	TEM-1 control (pI 5.4)
Lane 3	ROB-1 control (pI 8.1)

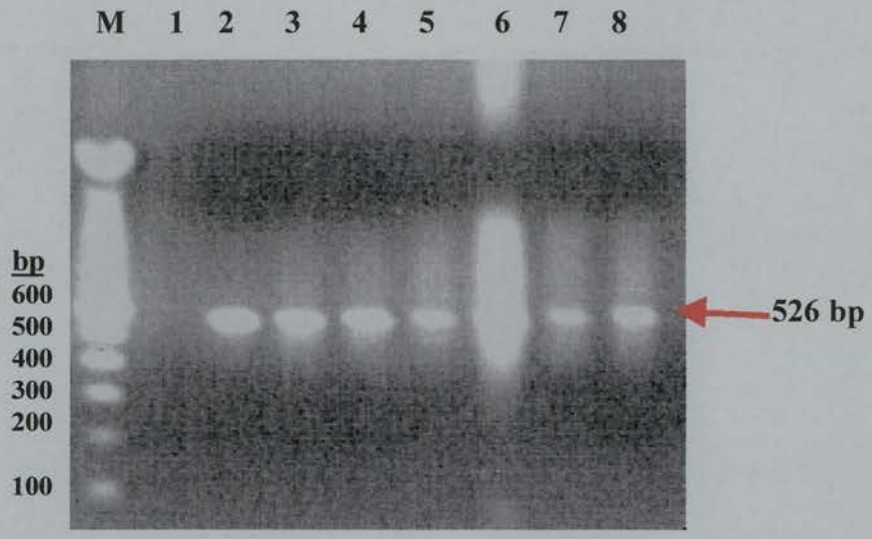
7.3.3. Identification of TEM-1-Derived β-lactamases by PCR

A total of 46 amoxycillin-resistant *H. influenzae* isolates were tested for the presence of bla_{TEM-1} type enzyme gene by PCR. According to the IEF results, 37 of these isolates were β-lactamase producers and the remaining 9 isolates were β-lactamase-negative.

Oligonucleotide primer pairs allowed amplification and isolation of 526 bp bla_{TEM-1} PCR products (Figure 7.2). All 37 amoxycillin-resistant β-lactamase-positive isolates, identified by nitrocephin spot test, produced the bla_{TEM-1} PCR product (Table 7.3). Interestingly, 6 of the 9 amoxycillin-resistant β-lactamase-negative isolates, missed by nitrocephin spot test, also produced the bla_{TEM-1} PCR product (Table 7.3). This could be explained that these isolates carry the bla_{TEM-1} gene responsible for β-lactamase production, but the gene is switched off, or the β-lactamase produced is so weak that can not be detected by nitrocephin spot test.

From the PCR results we can conclude that, in the 46 amoxycillin-resistant strains, 94% (43) possessed the TEM-1 β-lactamase gene and of these, 80% have active β-lactamase activity capable of conferring resistance.

Figure 7.2. Agarose gel showing amplified *bla*$_{TEM-1}$ PCR products (526 bp) from TEM-1 β-lactamase-positive amoxycillin-resistant *H. influenzae* clinical isolates



Lane M	100 bp lambda DNA ladder molecular marker
Lane 1	β-lactamase-negative *H. influenzae* control strain
Lane 2-8	*bla*$_{TEM-1}$ PCR products from amoxycillin-resistant β-lactamase-positive *H. influenzae* clinical isolates

7.3.4. Pulsed-Field Gel Electrophoresis (PFGE) Profiles

A total of 46 amoxycillin-resistant (and of these, 9 were also coamoxiclav-resistant) *H. influenzae* isolates were genotyped by PFGE (Figure 7.3 & 7.5). Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that amoxycillin-resistant isolates susceptible to clavulanic acid showed little genetic relatedness. In contrast, among coamoxiclav-resistant strains two major groups were identified. PFGE patterns were classified into groups alphabetically.

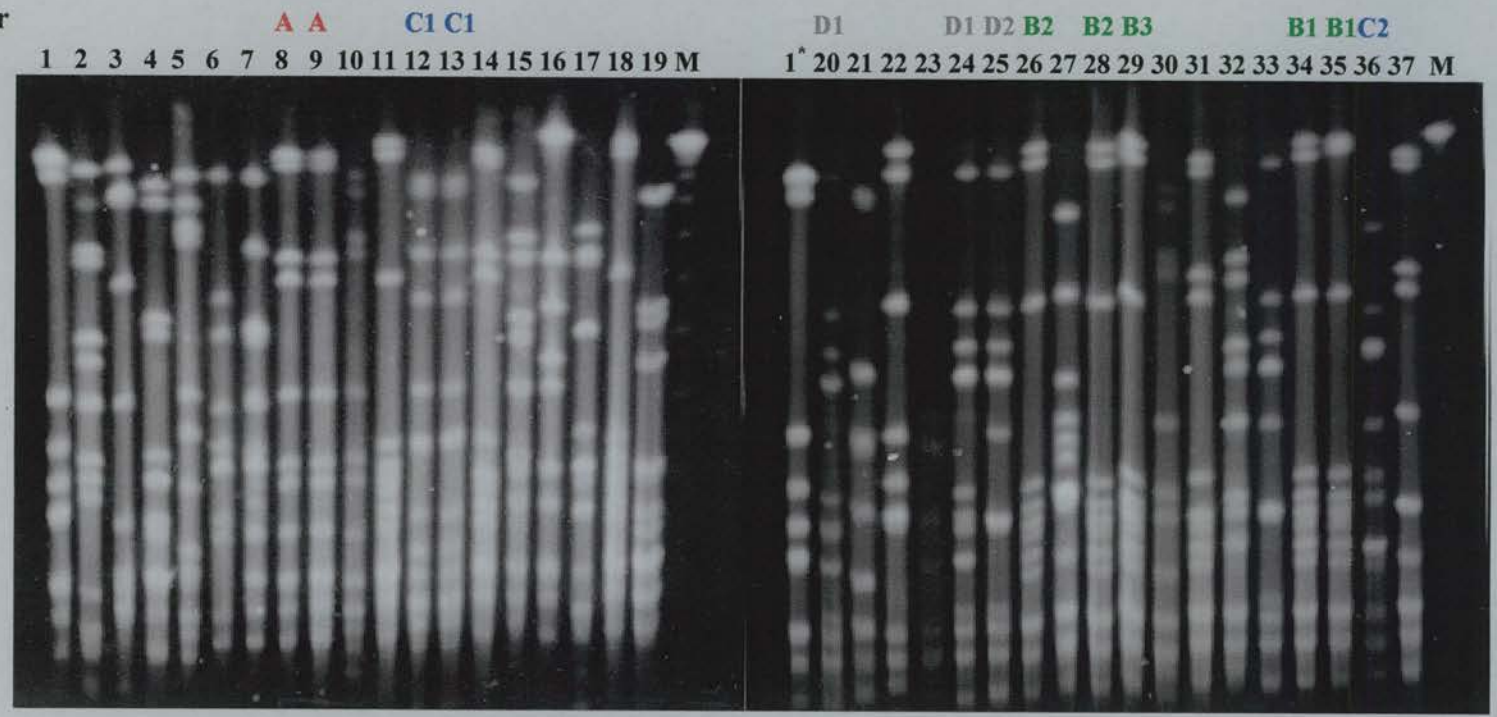
Amoxycillin-Resistant Co-amoxiclav-susceptible Isolates

The PFGE patterns of 37 isolates from the UK are shown in Figure 7.3. PFGE fingerprinting of the 37 isolates analysed by Gelcompar revealed that only 10 of the isolates were identical. Taking a cut off value of 80% similarity produced 4 clusters of isolates (A to D) within 13 strains from a total of 37 strains examined (Table 7.3). The remaining 24 isolates were unrelated and demonstrated 24 distinct types.

A dendrogram was constructed to show the degree of relatedness among the strains of clusters from the UK (Figure 7.4). Cluster A included two identical isolates from the same hospital both of which had the same amoxycillin MICs of 64 mg/L (RIE-238 & RIE-239). Cluster B included 5 isolates and were subtyped BI (RIE-195 & RIE-237), B2 (RIE-544 & RIE-547) and B3 (WH-621). Interestingly, all isolates in cluster B were from the same hospital, except WH-621, and have the same amoxycillin MICs of 64 mg/L. Cluster C included 3 isolates. Two isolates, LRI-319 & BSH-336, have the same PFGE patters but were from different geographic locations and have different amoxycillin MICs. They were subtyped as C1. The remaining isolate, B-410, was subtyped C2. Cluster D, with similar profile to cluster C, included 3 isolates. Two isolates, L-449 & RIE-506,

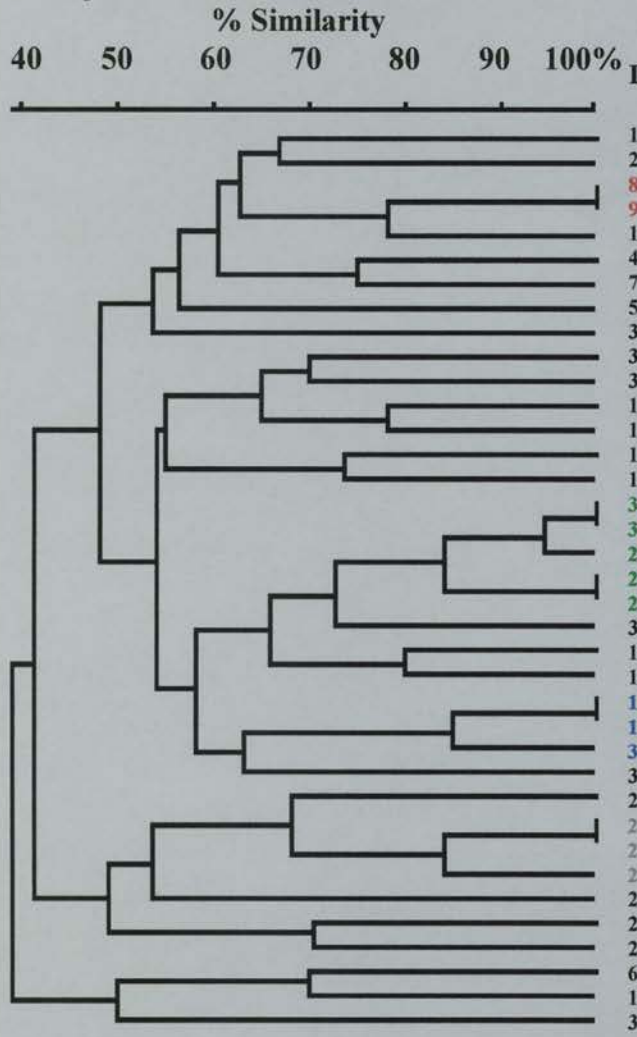
have the same PFGE patterns but were from different geographic locations and have different amoxycillin MICs. They were subtyped as D1. The remaining isolate, RIE-513, was subtyped D2.

Figure 7.3. Pulsed-field gel electrophoresis of *Sma*I digested chromosomal DNA from 37 amoxycillin-resistant co-amoxiclav-susceptible *H. influenzae* clinical isolates from the UK



Lane M	Lambda DNA ladder marker
Lanes 1 & 1*	*H. influenzae* strain RIE-188 (repeated twice as a control for both gels)
Lanes 2-37	36 amoxycillin-resistant co-amoxiclav-susceptible *H. influenzae* isolates

Figure 7.4. Dendogram illustrating the relatedness of 37 amoxycillin-resistant co-amoxiclav-susceptible *H. influenzae* isolates from the UK



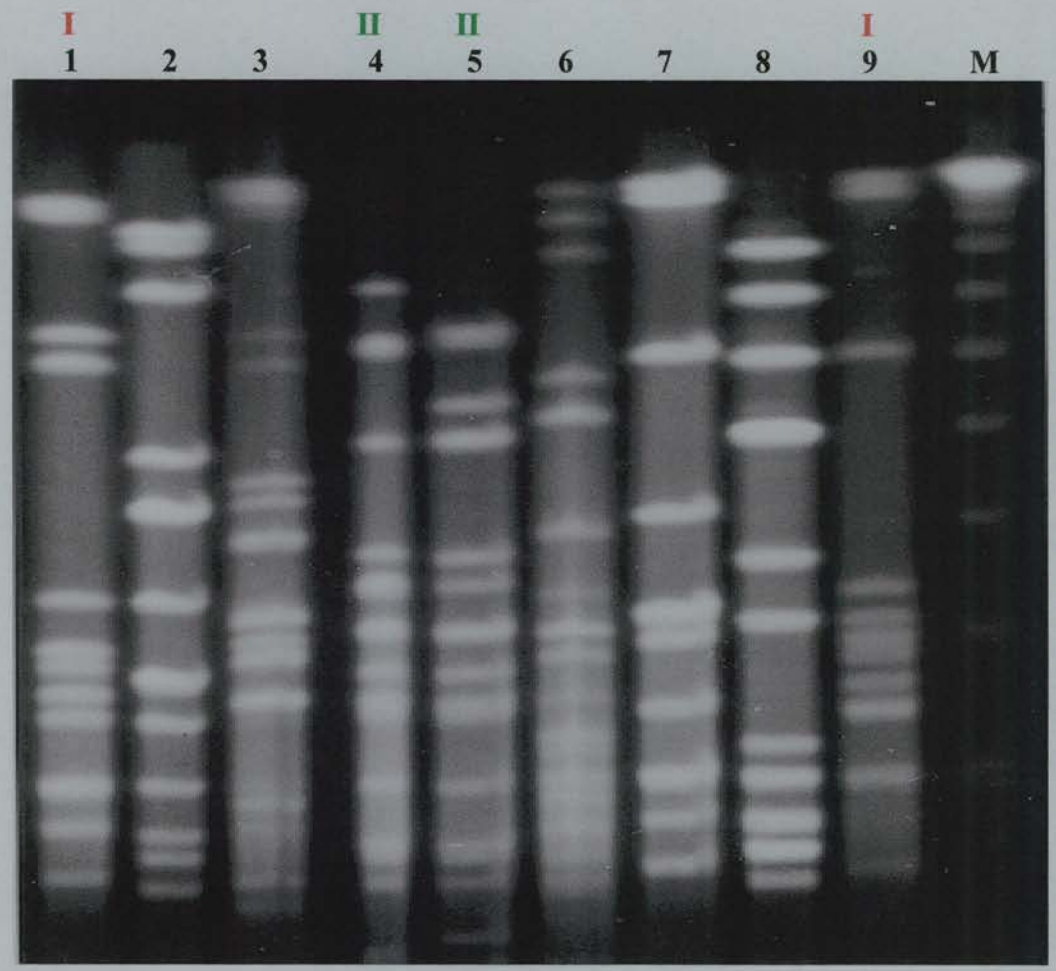
Lane	Strain	Amoxycillin MIC mg/L	TEM-1 IEF	TEM-1 PCR	PFGE
1					
27					
8	RIE-238	64	+	+	A
9	RIE-239	64	+	+	A
10					
4					
7					
5					
30					
32					
37					
15					
17					
16					
18					
34	RIE-195	64	-	+	B1
35	RIE-237	64	-	+	B1
29	WH-621	32	+	+	B3
26	RIE-544	64	+	+	B2
28	RIE-547	64	+	+	B2
31					
11					
14					
12	LRI-239	32	+	+	C1
13	BSH-336	8	+	+	C1
36	B-410	8	-	+	C2
33					
22					
20	L-449	4	+	+	D1
24	RIE-506	32	+	+	D1
25	RIE-513	32	+	+	D2
2					
21					
23					
6					
19					
3					

The scale measures similarity values. Taking a cut off value of 80% similarity, four clusters within 13 isolates were identified (typed A to D): Cluster A were classified into one subtype (A), **Cluster B** were classified into three subtypes (B1, B2 & B3), **Cluster C** were classified into two subtypes (C1 & C2), and **Cluster D** were classified into two subtypes (D1 & D2)

Amoxycillin-Resistant Co-amoxiclav-Resistant Isolates

Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that among co-amoxiclav-resistant isolates only two major groups of strains were identified (Figure 7.5 & 7.6). One group were TEM-1 producers and exhibited high MICs to amoxycillin (>8 mg/L) which were reduced by the addition of clavulanic acid. Taking a cut off value of 80% similarity produced one cluster of isolates (I) within 2 strains (RIE-191 & AH-763) from total of 5 strains within this group. The second group had the same MIC to amoxycillin as to co-amoxiclav and showed no evidence of β-lactamase production (Figure 7.6). Cluster analysis produced one cluster of isolates (II) within 2 strains (RIE-233 & FL-258) from total of 4 strains within this group. Neither group was restricted to isolates from the same geographic area.

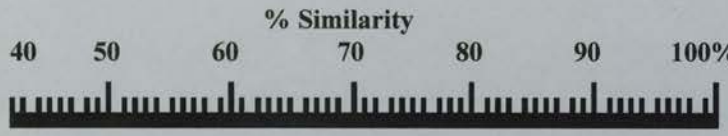
Figure 7.5. Pulsed-field gel electrophoresis of *Sma*I digested chromosomal DNA from 9 co-amoxiclav-resistant *H. influenzae* isolates from the UK



Lanes 1-9 9 co-amoxiclav-resistant *H. influenzae* isolates: RIE-191, LRI-213, LRI-216, RIE-233, FL-258, RIE-502, WH-624, AH-742 and AH-763, respectively.

Lane M Lambda DNA ladder marker

Figure 7.6. Dendrogram illustrating the relatedness of 9 co-amoxiclav-resistant *H. influenzae* isolates from the UK



Lane	Strain	Amxoxycillin MIC mg/L	Co-amoxiclav MIC mg/L	TEM-1 IEF	TEM-1 PCR	PFGE
1	RIE-191	64	4	+	+	I
9	AH-763	64	4	+	+	I
7	WH-624	16	4	+	+	
3	LRI-216	64	2	+	+	
6	RIE-502	16	4	-	+	
4	RIE-233	2	2	-	-	II
5	FL-258	2	2	-	-	II
8	AH-742	2	2	-	+	
2	LRI-213	4	4	-	-	

The scale measures similarity values. Taking a cut off value of 80% similarity, two clusters within 4 isolates were identified (typed I & II):Cluster I included two strains: RIE-191 and AH-763 and **Cluster II** included two strains: RIE-233 and FL-258.

Table 7.3. Amoxycillin and co-amoxiclav MICs of amoxycillin-resistant and co-amoxiclav-susceptible and -resistant *H. influenzae* isolates, PFGE and β-lactamase IEF and PCR results

Serial No.	Strain	PFGE	MIC (mg/L)		TEM-1 β-Lactamase	
			Amoxycillin	Co-amoxiclav	IEF	PCR
Amoxycillin-resistant, co-amoxiclav-susceptible *H. influenzae* isolates						
1	RIE-188		64		+	+
2	RIE-189		64		+	+
3	LRI-205		16		+	+
4	LRI-211		4		+	+
5	LRI-218		4		+	+
6	RIE-231		32		+	+
7	RIE-234		4		+	+
8	RIE-238	A	64		+	+
9	RIE-239	A	64		+	+
10	BRI-244		4		+	+
11	BRI-246		4		+	+
12	LRI-319	C1	32		+	+
13	BSH-336	C1	8		+	+
14	BSH-341		2		+	+
15	BSH-348		8		+	+
16	LRI-362		4		+	+
17	LRI-363		2		+	+
18	WH-411		16		+	+
19	WH-414		4		+	+
20	L-449	D1	4		+	+
21	L-463		8		+	+
22	RIE-499		64		+	+
23	RIE-500		2		+	+
24	RIE-506	D1	32		+	+
25	RIE-513	D2	32		+	+

.....continue Table 7.3

Table 7.3. .....continue

Serial No.	Strain	PFGE	MIC (mg/L)		TEM-1 β-Lactamase	
			Amoxycillin	Co-amoxiclav	IEF	PCR
26	RIE-544	B2	64		+	+
27	RIE-546		64		+	+
28	RIE-547	B2	64		+	+
29	WH-621	B3	32		+	+
30	RIE-637		64		+	+
31	AH-748		32		+	+
32	AH-749		4		+	+
33	D-772		16		+	+
34	RIE-195	B1	64		-	+
35	RIE-237	B1	64		-	+
36	B-410	C2	8		-	+
37	RIE-587		2		-	+
Amoxycillin-resistant, co-amoxiclav-resistant *H. influenzae* isolates						
38	RIE-191	I	64	4	+	+
39	AH-763	I	64	4	+	+
40	WH-624		16	4	+	+
41	LRI-216		64	2	+	+
42	RIE-502		16	4	-	+
43	AH-742		2	2	-	+
44	RIE-233	II	2	2	-	-
45	FL-258	II	2	2	-	-
46	LRI-213		4	4	-	-

IEF Isoelectric foucsing, **PCR** Polymerase chain reaction, **MIC** Minimum inhibitory concentration, **PFGE** Pulsed-field gel electrophoresis

7.4. Discussion

β-lactams compounds are among the drugs of choice for treating respiratory infections caused by *H. influenzae*. However, the emergence of β-lactam resistant strains has influenced the therapeutic efficacy of these compounds. The most common mechanism of β-lactam resistance in *H. influenzae* is the production of an enzyme with β-lactamase activity. It is, therefore, essential that one can identify the production of β-lactamases by clinical isolates and have effective ways of distinguishing the different enzymes. This is necessary for epidemiologic surveys, predicting future resistance trends, and to ensure that patients receive the appropriate β-lactam or alternative therapy.

From 231 *H. influenzae* strains examined in this study, 16% produced detectable β-lactamase enzymes. This rate was similar to the Canadian (25%) (Matsumura *et al.*, 1995) and American (16.5%) (Jorgensen *et al.*, 1990) studies. Also the results showed that the prevalence of β-lactamase mediated amoxycillin resistance (18.6%) was comparatively higher than the 8.6% and 8.4% recorded in the studies conducted in the UK (Powell *et al.*, 1992) and Wales (Howard & Williams, 1988). To extend the antimicrobial spectrum, clavulanic acid was combined with amoxycillin (2:1), however; a high proportion (21%) of the β-lactamase-producing amoxycillin-resistant strains remained insensitive to amoxycillin. This may be explained by the hyper-production of β-lactamase enzymes in clinical strains which, in turn, is a threat to the effectiveness of the β-lactamase inhibitors currently administered for the treatment of β-lactamase-positive *H. influenzae* infections. TEM-1 production accounted for all the β-lactamase-positive strains isolated in this study. None of the strains contained enzymes identified as ROB-1 or VAT-1. In previous studies, TEM-1 and ROB-1 production accounted for approximately 96% and 7% of the β-lactamase-positive strains, respectively (Scriver *et*

al., 1994; Powell *et al.,* 1992). The isolation of a VAT-1 type enzyme has been reported only on rare occasions (Vali *et al.,* 1994; Shanahan *et al.,* 1996).

Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that amoxycillin-resistant isolates showed little genetic relatedness. However, few clusters of isolates have been identified. According to these results, it seems that the clonal propagation of these resistant strains does not occur as previously described *in S. pneumoniae* clinical isolates (Muñoz *et al.,* 1991).

In summary, amoxycillin resistance in *H. influenzae* throughout the UK is mediated by the TEM-1 enzyme which appears to have spread into a wide variety of different strains.

CHAPTER EIGHT

Analysis of Penicillin-Binding Proteins and Outer-Membrane Proteins of Amoxycillin-Resistant Co-Amoxiclav-Resistant Clinical Isolates of *H. influenzae*

8.1. Introduction

β-Lactamase enzymes play a major role in *H. influenzae* resistance to β-lactam antibiotics. However, there are increasing numbers of reports of *H. influenzae* strains that owe their β-lactam resistance to a mechanism other than the production of a β-lactamase. Changes in the penicillin-binding proteins (PBPs) and outer-membrane proteins (OMPs) can both result in β-lactam resistance (Malouin & Bryan, 1986; Reid *et al.*, 1987; Nikaido, 1989).

Ampicillin-resistant non-β-lactamase-producing *H. influenzae* strains (AMP^r β^-) are more difficult to detect because of their low incidence rate. Also they have lower-level resistance than do β-lactamase-producing strains (Mendelman *et al.*, 1986). The AMP^r β^- *H. influenzae* strains are genetically diverse and have been reported in the United States, Canada, the United Kingdom and New Zealand (Mendelman *et al.*, 1987). Their overall incidence in the United Kingdom increased from 2 % in 1981 to 4% in 1986 and to 5.8% in 1991 (Powell *et al.*, 1987; 1992). However, conventional methods for testing the susceptibility of *H. influenzae* to antibiotics are inoculum dependent and have been

shown to be unreliable (Mendelman *et al.*, 1986; Powell & Williams, 1988; Heelan *et al.*, 1992). It has also been suggested that the frequency of AMP^r β$^-$ *H. influenzae* strains are underestimated (Powell & Williams, 1988; Brown *et al.*, 1992). The usual disk diffusion method with a 10- or 25-μg amoxycillin disk does not permit the detection of this mode of resistance. Detection of this kind of resistance (diameter of inhibition, <19 mm, corresponding to an MIC of ≥2 mg/L) is only obtained with a 2-μg ampicillin disk (BSAC, 1998).

PBPs are targets for β-lactams. Binding of β-lactam antibiotics to PBPs leads to cell lysis, death or growth arrest (Tomasz, 1979; Tipper, 1985). Alterations in PBPs profile is one of the mechanisms that develops resistance to β-lactam compounds. The number, amount and size of PBPs as well as their affinity for β-lactam antibiotics vary from species to species (Makover *et al.*, 1981). Among the 10 *Haemophilus* species isolated from humans, only *H. influenzae* and *H. aegyptius* appear to have similar PBPs (Mendelman & Serfass, 1988). Ampicillin-susceptible strains of *H. influenzae* have a relatively homogeneous pattern of 8 PBPs (Mendelman *et al.*, 1990). In contrast, AMP^r β$^-$ *H. influenzae* strains have heterogeneous PBP profiles (Mendelman *et al.*, 1990).

Although it is likely that the alteration of PBPs is the most frequent cause of AMP^r β$^-$ in *H. influenzae* strains, decreased outer-membrane permeability has also been reported. Burns and Smith (1987) found that some *H. influenzae* strains resistant to β-lactams lacked a 40 kDa major porin. However, the PBPs of that particular isolate were not investigated. Outer-membrane protein profiles of both capsulated and non-capsulated *H. influenzae* strains, have proved to be diverse and variant (Loeb & Smith, 1980). Many workers have suggested that AMP^r β$^-$ in *H. influenzae* strains may only be caused by altered PBPs (Clairoux *et al.*, 1992), however, Reid *et al.* (1987) implied that OMP profile differences exist between ampicillin-susceptible and AMP^r β$^-$ *H. influenzae* strains of the same biotype. However, no specific protein change accounted for this finding.

During the study of amoxycillin-resistant *H. influenzae* strains, a group of 9 strains were identified as also co-amoxiclav-resistant (see Chapter 3). In the current study these 9 strains were further examined for their PBP and OMP profiles.

8.2. Materials & Methods

8.2.1. Bacterial Strains

Nine co-amoxiclav-resistant *H. influenzae* isolates were examined for their OMPs and PBPs profiles. An amoxycillin-susceptible *H. influenzae* isolate was also included as control.

8.2.2. Alterations in Outer-Membrane Proteins (OMPs)

OMPs Preparation

Membrane fractions were prepared by a modification of the methods of Reid *et al.*, (1987). Volumes of 10 ml of overnight cultures were inoculated into 90 ml of Heart Infusion Broth supplemented with 10 μg/ml Haemin and 10 μg/ml NAD and incubated at 37°C, with shaking at 200 rpm for 4 h, to yield mid-log phase cells. Cells were then harvested by centrifugation at 6,000 g and 4°C for 10 min and the pellet resuspeneded in 10 ml of cold sodium phosphate buffer (50 mM, pH 7.0). The suspensions were sonicated (MSE Soniprep 150, MSE Instruments, Crawley, Sussex) at 8 μm for 6 cycles of 30 sec each in an ice bath, separated by 30 sec cooling periods. They were then centrifuged at 2,500 g and 4°C for 10 min to remove debris and unbroken cells. The supernatants were retained and the cell-envelopes harvested by centrifugation at 15,600 g and 4°C for 30 min to sediment the cell membrane. The inner membranes were solubilised by the addition of 2 ml of 2% *n*-lauroylsarcosine. Then the OMPs were sedimented by ultracentrifugation at 40,000 g for 1 h at 4°C, resuspended in 1 ml MilliQ water, dispensed into aliquots and stored at -20°C until required.

Protein Concentrations in the OMP Preparations

OMPs were diluted in MilliQ water to a concentration of 1 mg/ml. The Protein concentrations in the OMP preparations were determined by the method of Waddell and Hill (1956). The samples were diluted in sterile distilled water and their absorbance was measured at wavelengths of 215 and 225 nm on a Perkin-Elmer UV/Vis Lambda II Spectrophotometer. The protein concentration was determined by reference to a previously prepared standard curve.

Gel Electrophoresis

After adjusting their protein concentration to 1 mg/ml, 50 µl volumes of the membrane extracts were mixed with 50 µl of loading buffer comprising 20% glycerol, 0.005% bromophenol blue, 4% sodium dodecyl sulphate (SDS) and 5% β-mercaptoethanol in 0.25 M Tris-HCl (pH 6.8), and boiled for 5 min. After cooling, samples were loaded onto 12% Tris-HCl SDS polyacrylamide Mini-Protean Ready gel (BioRad, UK) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the Mini-Protean II System (Bio-Rad, UK) at 200 V constant voltage for 30 min. The running buffer consisted of 0.025 M Tris-HCl, 0.19 M glycine and 0.1% SDS. A low range molecular weight standard (Bio-Rad, UK) was also loaded in the gel. After electrophoresis, the gel was removed from the apparatus and stained overnight with Coomassie blue R-250 staining solution (Sigma, UK), then destained in several changes of distilled water before being photographed.

8.2.3. Alterations in Penicillin Binding Proteins (PBPs)

PBPs Preparation

Membrane fractions were prepared by a modification of the methods of Reid *et al.*, (1987). Volumes of 100 µl of overnight cultures were inoculated into 400 ml amounts of Heart Infusion Broth (Oxoid, UK) supplemented with 10 µg/ml Haemin and 10 µg/ml NAD and incubated at 37°C, with shaking at 200 rpm for 4 h, to yield mid-log phase cells. The cells were then harvested at 5,000 g for 20 min at 4°C, washed with 10 ml ice-cold 20 mM sodium phosphate buffer (pH 7.0), and resuspended in 10 ml of the same buffer containing 140 mM β-mercaptoethanol. The resulting suspensions were subjected to sonication (MSE Soniprep 150, MSE Instruments, Crawley, Sussex) at 18 µm amplitude for 6 cycles of 30 sec each in an ice bath, then centrifuged at 1,500 g for 15 min at 4°C to remove debris. The supernatants were retained and the membrane particles were deposited by centrifugation at 150,000 g for 30 min at 4°C and resuspended in 0.5 ml of 20 mM sodium phosphate buffer (pH 7.0). These suspensions were then dispensed in aliquots and stored at -70°C until required. The PBPs were diluted in MilliQ water to a concentration of 5 mg/ml as described above.

Direct PBP Labelling

Membrane fractions, in 90 µl volumes, were thawed slowly and mixed with 10 µl amounts of 330 mg/L ^{14}[C] benzyl penicillin (Amersham Life Science) in phosphate buffer (pH 7.0) and incubated at 30°C for 10 min. Ten-microlitre amounts of non-radioactive benzylpenicillin solutions, 60 mg/ml, in phosphate buffer (pH 7.0) were then added followed by 10 µl of 10% *n*-lauroylsarcosine. The mixtures were vortexed briefly, held at room temperature for 20 min and the preparations were then ready for electrophoresis.

Gel Electrophoresis

The above preparations were mixed with 50 µl of loading buffer comprising 20% glycerol, 0.005% bromophenol blue, 4% sodium dodecyl sulphate (SDS) and 5% β-mercaptoethanol in 0.25 M Tris-HCl (pH 6.8), and boiled for 3 min. After cooling, samples were loaded onto the gel and run exactly as described in the section on OMP gel electrophoresis (see section 8.2.2). After running, the gel was stained and destained before being photographed.

Fluorography

The destained gel was impregnated with aqueous scintillant, Amplify (Amersham Life Science), for 1 h, then transferred on to Whatman no.17 card and dried under vacuum using a gel dryer (BioRad, UK). The dried gel was exposed to X-ray film (Kodak XOMAT AR) at -70°C for 3 months before developing.

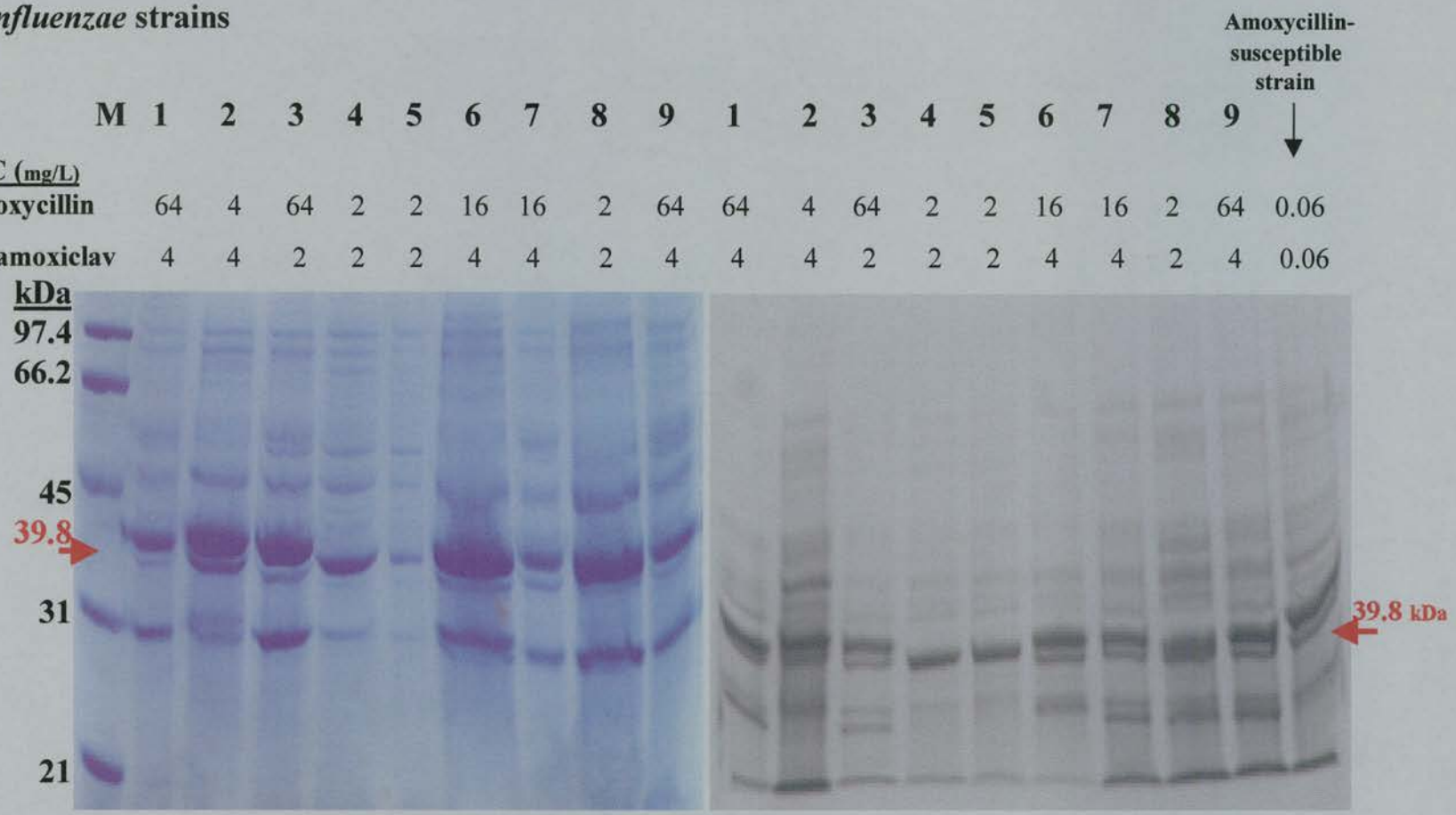
8.3. Results

8.3.1. Outer-Membrane Proteins (OMPs) Profiles

The OMP profiles of the 9 amoxycillin-resistant co-amoxiclav-resistant strains were compared to that of an amoxycillin- and co-amoxiclav-susceptible strain. All strains produced a limited number of major OMPs with molecular sizes between 31 and 45 kDa, together with various other minor protein components (Figure 8.1).

Comparison of the OMPs profiles revealed that similar patterns were observed in most of the co-amoxiclav-resistant strains compared with the susceptible strain (Figure 8.1). However, two strains, RIE-233 and FL-258, lacked the 39.8 kDa band which was present in all the other strains (Figure 8.1 & 8.2). Interestingly, these two isolates have the same MIC results for amoxycillin and co-amoxiclav (2 mg/L), are non-β-lactamase producers, and gave similar PFGE patterns as shown in Table 8.1. Both isolates were from different geographical locations. The lacking of 39.8 kDa band in these two isolates suggest a correlation of this band with amoxycillin and co-amoxiclav reduced susceptibility.

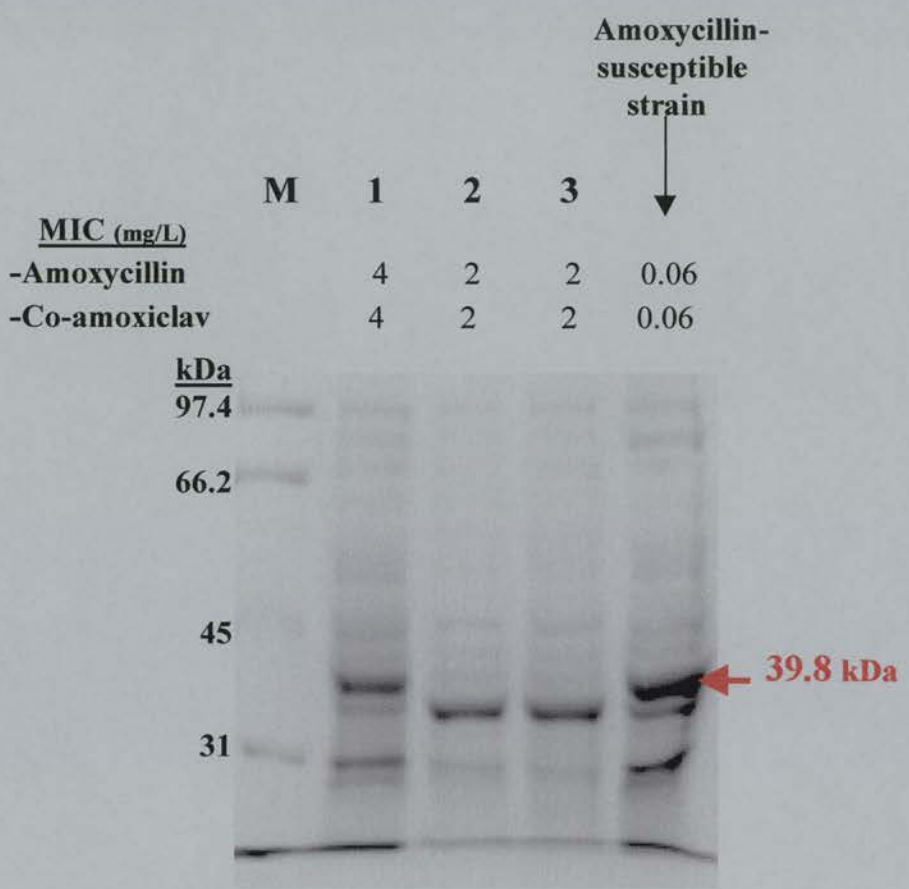
Figure 8.1. SDS-PAGE of outer-membrane proteins (OMPs) of 9 co-amoxiclav-resistant *H. influenzae* strains



Lane M Molecular weight marker

Lanes 1-9 Nine co-amoxiclav-resistant *H. influenzae* strains: RIE-191, LRI-213, LRI-216, RIE-233, FL-258, RIE-502, WH-624, AH-742 and AH-763, respectively.

Figure 8.2. SDS-PAGE of outer-membrane proteins (OMPs) of β-lactamase-negative co-amoxiclav-resistant *H. influenzae* strains



Lane M Molecular weight marker

Lanes 1-3 *H. influenzae* strains LRI-213, RIE-233 and FL-258, respectively.

8.3.2. Penicillin Binding Proteins (PBPs) Profiles

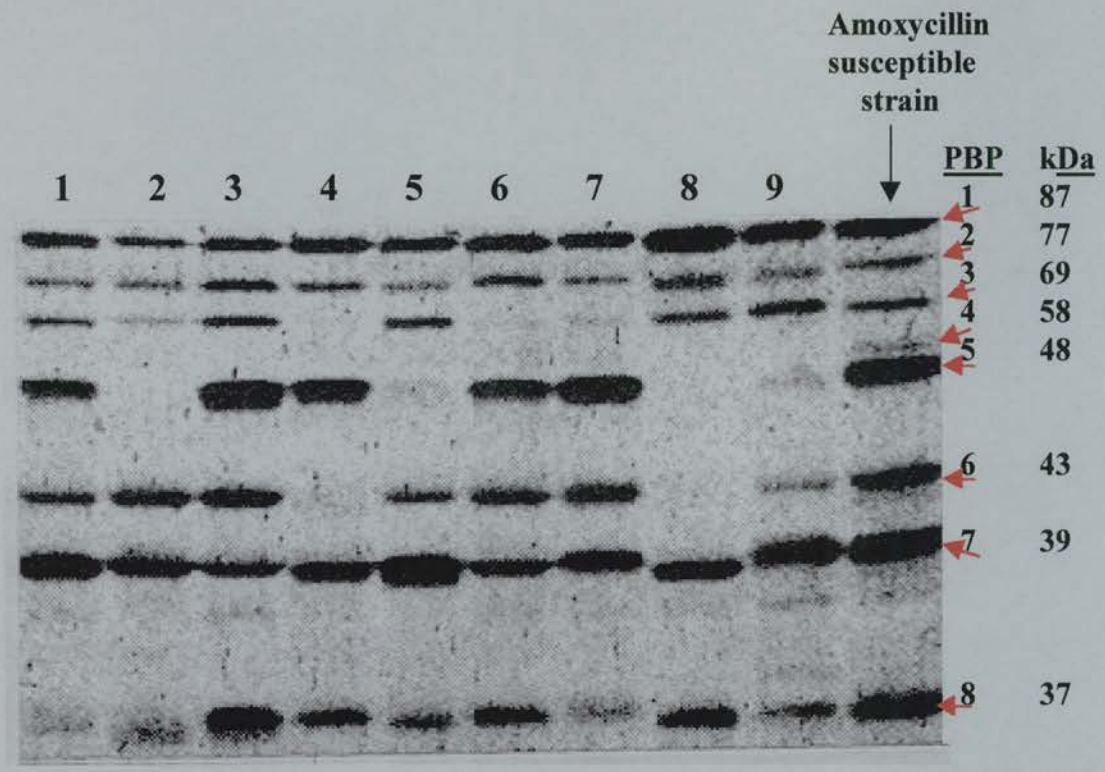
The PBPs of the 9 amoxycillin-resistant co-amoxiclav-resistant isolates and the susceptible strain were compared. Eight distinct PBPs were detected in the amoxycillin-susceptible strain examined, with molecular weights between 37 and 87 kDa (Figure 8.3).

In contrast, the 9 co-amoxiclav-resistant strains tested exhibited marked heterogenicity in patterns (Figure 8.3). PBPs 1 (87 kDa), 2 (77 kDa), 3 (69 kDa), 7 (39 kDa) and 8 (37 kDa) were present in all 9 isolates and variation in electrophoretic mobility as well as in the amount of penicillin bound by certain PBPs was noted. However, among co-amoxiclav-resistant strains, penicillin binding to the PBPs of certain isolates was either markedly reduced or absent, as in PBP4 (58 kDa) and PBP5 (48 kDa) in lanes 2 and 8; and PBP6 (43 kDa) in lanes 4 and 8 (Figure 8.3).

Table 8.1 shows a summary of the possible mechanisms of resistance to amoxycillin and co-amoxiclav in *H. influenzae* isolates.

Figures 8.3. Fluorography of PBPs of co-amoxiclav-resistant *H. influenzae* strains

The numbering system follows Makover *et al.* (1981)



Lanes 1-9 co-amoxiclav-resistant *H. influenzae* strain RIE-191, LRI-213, LRI-216, RIE-233, FL-258, RIE-502, WH-624, AH-742 and AH-763, respectively.

Table 8.1. Summary of co-amoxiclav-resistant *H. influenzae* strains resistance mechanisms

Strain	Amoxycillin* MIC (mg/L)	Co-amoxiclav* MIC (mg/L)	PFGE pattern	TEM-1		Changes in		Possible mechanisms of resistance
				IEF	PCR	OMP	PBP	
RIE-191	64	4	I	+	+	-	-	β-lactamase
AH-763	64	4	I	+	+	-	-	β-lactamase
WH-624	16	4		+	+	-	-	β-lactamase
LRI-216	64	2		+	+	-	-	β-lactamase
RIE-502	16	4		-	+	-	-	β-lactamase
RIE-233	2	2	II	-	-	+	+	OMP/PBP
FL-258	2	2	II	-	-	+	-	OMP
AH-742	2	2		-	+	-	+	β-lactamase/PBP
LRI-213	4	4		-	-	-	+	PBP

PFGE pulsed-field gel electrophoresis, **PCR** polymerase chain reaction, **IEF** isoelectric focusing, **OMP** outer-membrane protein, **PBP** penicillin-binding protein, (+) difference from amoxycillin-susceptible strain

* Resistant breakpoint for amoxycillin and co-amoxiclav is ≥1 mg/L

8.4. Discussion

H. influenzae have acquired a wide range of defence mechanisms which may protect them from the actions of antibiotics. However, one way of overcoming the resistance caused by these defence systems is to understand the biochemical mechanisms. When these mechanisms are fully understood and combined with information on the prevalence, strategies may be designed for maximising the usefulness of antimicrobial agents whilst minimising the frequency of antibiotic resistance.

Amoxycillin resistance in *H. influenzae* is mainly caused by β-lactamase production. However, intrinsic mechanisms entailing target modification (PBP alterations) and/or impermeability (OMP alterations) have also been described (Reid *et al.*, 1987).

Ampicillin-resistant non-β-lactamase-producing *H. influenzae* strains (AMP^r β^-) are not frequently encountered. Depending on which breakpoints are considered, the incidence of these strains varies. The frequencies of occurrence of resistant strains are <1 to 2.5% (MICs ≥2 mg/L) in Canada and the United States (Barry *et al.*, 1993; Clairoux *et al.*, 1992; Doern *et al.*, 1988), 5 to 7% (MICs ≥1 mg/L) in the United Kingdom (Reid *et al.*, 1987; Powell *et al.*, 1990; Powell & Livermore, 1990), <1 to 7% (MICs ≥1 or ≥2 mg/L) in Australia (Bell & Plowman; 1980; Collignon *et al.*, 1992), 1% (MICs ≥2 mg/L) and 0.3% (MICs ≥4 mg/L) in an international study (Kayser *et al.*, 1990). This variability in the incidence of AMP^r β^- illustrates the difficulties encountered in highlighting this kind of resistance.

In the current study, in common with other investigators (Serfass *et al.*, 1986; Reid *et al.*, 1987; Mendelman *et al.*, 1990), eight PBPs were detected in a fully-susceptible *H. influenzae* strain. However, different PBP numbering schemes have been used by different workers (Mendelman *et al.*, 1990). In the present study, 3 of the nine co-amoxiclav-resistant *H. influenzae* strains showed differences in the PBPs profile relative to the amoxycillin-susceptible control strain.

Also the absence of OMP band at 39.8 kDa in two β-lactamase-negative co-amoxiclav-resistant strains (RIE-233 and FL-258) but not in the amoxycillin-susceptible control strains, suggested an association between the absence of this protein and co-amoxiclav resistance.

The OMP and PBP results show that several mechanisms, either acting individually or in combination, are implicated in amoxycillin and co-amoxiclav resistance in *H. influenzae*. It is thus apparent that β-lactamase enzymes are not the sole mechanisms of amoxycillin resistance in *H. influenzae* and that resistance may also result from PBP and permeability alterations.

CHAPTER NINE

General Discussion

9.1. Introduction

The development of antibiotics has undoubtedly been one of the greatest achievements of modern medicine. However, increasing bacterial resistance to these agents is compromising their usefulness and there is genuine concern that we may be approaching the end of the antibiotic era. There are two approaches to overcome this, one is a need to develop new agents and the other is a better understanding of how resistance to current antibiotics is developing and spreading. This thesis attempts to reveal the latter in common lower respiratory pathogens.

β-Lactams account for approximately 50% of global antibiotic consumption (Livermore, 1998) and this heavy usage exerts considerable selection for resistance arising via (i) modification and addition of the normal penicillin-binding proteins (PBPs) the latter allowing bypassing of the normal PBPs, (ii) impermeability of the gram-negative organism outer membrane and (iii) production of β-lactamases (Livermore, 1998). PBP modification and bypassing are the most important mechanisms of resistance in gram-positive cocci (except β-lactamase-producer *Staphylococcus aureus* strains), whereas β-lactamases are pre-eminent in gram-negative species.

One of the major uses of β-lactams is to treat *S. pneumoniae* and *H. influenzae* lower respiratory infections. However, resistance to β-lactams is increasing in the UK (Goldsmith *et al.*, 1997; Powell *et al.*, 1992). To establish if this increase results from

spread of a small number of resistant strains or from a wider emergence of resistance, the molecular type and mechanisms of resistance in penicillin-resistant *S. pneumoniae* and amoxycillin-resistant *H. influenzae* clinical isolates were examined. In an attempt to establish if alternative therapies might be viable, the sensitivity and resistance of penicillin-resistant *S. pneumoniae* to fluoroquinolones was also investigated.

To be a useful epidemiologic tool, a typing system must give an unambiguous result for each isolate (typeability), give the same result each time the same isolate is tested (reproducibility), and differentiate among epidemiologically unrelated strains (discriminatory power) (Weber *et al.*, 1997). The earliest typing methods used for epidemiologic purposes were phenotypic methods. Phenotypic methods can detect characteristics expressed by microorganisms, in response to antibiotics or other inhibitors, often as a product of one of their enzymes, or even as a protein antigen on their cell surface (Grandsen *et al.*, 1985; Mulligan *et al.*, 1988; Boyce *et al.*, 1992). Phenotypic methods rely on gene expression and are not as stable as DNA-based methods. In addition, phenotypic methods tend to categorize isolates together in large groups and thus do not discriminate between strains as efficiently as DNA-based methods (Weber *et al.*, 1997). Genotypic typing methods evaluate differences at the DNA level and probably should be more commonly used because of their greater discrimination than phenotypic methods (Hall, 1998). In addition, in theory at least all isolates should be typeable by tests evaluating the chromosomal DNA and the results should be reproducible, which has not been the case for phenotypic methods (Weber *et al.*, 1997).

9.2. The Level of Antibiotic Resistance in Lower Respiratory Pathogens

S. pneumoniae

In the current study, the rate of penicillin resistance in pneumococcal isolates from selected laboratories in the UK was 11.4%. Significant and rising levels of pneumococcal penicillin resistance have now been reported from several parts of the UK. Nair (1988) reported low-level penicillin resistance amongst 4% of 100 pneumococcal strains tested in Hammersmith, London. Ridgway *et al.* (1991) examined 915 strains isolated between 1987 and 1989 in the Merseyside area and showed that the prevalence of resistance rose from 1.4% to 2.5% during this time. A subsequent study in 1993 found 48% of 521 pneumococci to be resistant to at least one antibiotic (Ridgway *et al.*, 1995) and in 1995 the level of penicillin resistance had risen to 7.5% (Allen & Anson, 1996). In Newham, London, Wilson *et al.*, (1996) found 12% of isolates in 1994-1995 to be similarly resistant. Data from the Ptolemy Project multi-centre survey (Felmingham *et al.*, 1996) between 1994-1995 showed an overall UK prevalence of amoxycillin resistance 6.8%. However, these figures varied from 3.6% in Bristol and 5% in Bangor, up to 10% in Newcastle, 10.7% in Notingham, 11.9% in Belfast and 20% in Liverpool. In contrast no penicillin-resistant pneumococci were reported from centres in Glasgow and London. The number of laboratories in England and Wales reporting the isolation of penicillin-resistant pneumococci to the Central Public Health Laboratory (CPHL) increased four-fold between 1987 and 1991 (George *et al.*, 1992) and a recent study from this reference laboratory showed 2.5-fold increase between 1990 and 1995 (Johnson *et al.*, 1996). Clearly penicillin-resistant pneumococci are increasing in prevalence in the UK, and this increase appears likely to continue.

High proportion of penicillin-resistant pneumococci are also resistant to erythromycin (Schutze *et al.*, 1994; Boswell *et al.*, 1996). Erythromycin resistance was detected in 12.9% of the pneumococcal isolates in this thesis. The CPHL has reported a three-fold increase in erythromycin resistance in pneumococci from point prevalence studies

performed in 1990 and 1995 (Johnson *et al.*, 1996). The frequency of erythromycin resistance in pneumococci in 1995 was estimated to be 8.6%, thus the increase in pneumococcal erythromycin resistance seems to have paralleled that of penicillin and indeed many pneumococci are resistant to both antibiotics. In this thesis, the associated resistance to erythromycin, tetracycline and trimethoprim amongst penicillin-resistant pneumococci were 56%, 46% and 71%, respectively. In contrast, the associated resistance to erythromycin, tetracycline and trimethoprim amongst penicillin-sensitive pneumococci were 8%, 8% and 32%, respectively. This high level of associated resistance amongst penicillin-resistant pneumococci is cause of concern.

H. influenzae

In the current study, the prevalence of β-lactamase-mediated amoxycillin resistance (18.6%) was comparatively higher than the 6.2%, 8.6% and 8.4% recorded in the studies conducted in UK in the 1986 and 1991 (Powell *et al* 1987; 1992) and in Wales (Howard & Williams, 1988), respectively. However, this rate of resistance was similar to the overall prevalence of β-lactamase-positive *H. influenzae* (19.2%) reported from the Alexander Project Group in 1995 (Schito *et al.*, 1997). Variations in testing methodologies carried out in this study compare with other studies may have affected the results of the sensitivity testing, therefore, it is likely that resistance patterns may have been under-estimated. To extend the range of susceptible bacteria clavulanic acid was combined with amoxycillin (2:1); however, a high proportion (14%) of the β-lactamase-producing amoxycillin-resistant strains remained insensitive to amoxycillin in the presence of clavulanic acid. This may be explained by the hyper-production of β-lactamase enzymes in clinical strains which, in turn, is a threat to the effectiveness of the β-lactamase inhibitors currently administered for the treatment of β-lactamase-positive *H. influenzae* infections. It may also be due to multiple-resistance mechanisms where there are permeability mutations or altered PBPs in addition to β-lactamase production.

The incidence of amoxycillin-resistant β-lactamase-negative strains was 6.5%, slightly higher than those reported in the UK in the 1986 and 1991 (4% and 5.8%, respectively) (Powell *et al.*, 1987; 1992), but this difference is probably not statistically significant. The overall rate of non-β-lactamase amoxycillin-resistance appears stable at around 5%.

Overall, 10% of the isolates were resistant to cefaclor and 5.2% to cefuroxime. Resistance to cefaclor has increased markedly over the past years compared to the 1.6% recorded in Wales in 1988 (Howard & Williams, 1988). The differences in the incidence of cefaclor resistance is either due to the increased usage of the drug or may be related to the introduction of amoxycillin plus clavulanic acid as the current results suggested some degree of cross-resistance.

The incidence of tetracycline resistance was 3.9%, slightly higher than that reported in the UK in 1986 and 1991 (2.7% and 1.4%, respectively) (Powell *et al.*, 1987; 1992). Resistance to trimethoprim has increased further to 9.6%, continuing the trend demonstrated previously between 1986 (4.2%) and 1991 (6.4%) (Powell *et al* 1987; 1992). There were no strains in the current study that had reduced sensitivity to ciprofloxacin.

9.3. The Relationship between Classical Serotyping and Molecular Typing of Penicillin-Resistant *S. pneumoniae* Strains

Conclusions on Typing Techniques

The spread of antibiotic resistance and the development of new vaccines have focused attention on the epidemiology of *S. pneumoniae* over recent years. While serotyping and the determination of antibiotic resistance remain primary methods for characterising pneumococci, molecular typing, such as PFGE, can add greater discrimination and

complementary information. In this study, the usefulness of capsular serotyping and PFGE macrorestriction with *Sma*I enzyme of pneumococcal chromosomal DNA was investigated to establish if penicillin resistance was the result of clonal spread and to determine whether isolates of a given serotype were more closely related to each other than to isolates of other serotypes. The results obtained by PFGE separations of restriction fragments of *S. pneumoniae* DNA indicate that there is no correlation demonstrated between PFGE profiles and serotyping. In a previous study, DNA fingerprint differences were found among serotypes, with significantly greater similarity among strains of a given serotype (Viering & Fine, 1989). The data in this thesis clearly show that different capsular types can be genetically closely related whereas strains with the same serotype may show quite different PFGE fingerprints and presumably, greater diversity of genetic backgrounds. The results presented here confirm that capsular type may not a good criterion for genetic relatedness as reported in several previous studies (Sibold *et al.*, 1992; Lefevre *et al.*, 1993; Hall *et al.*, 1996). The distinguishable PFGE patterns in strains with the same serotype, suggests either that serotype is not closely linked to the genetic relatedness of the isolate or that population is not truly clonal in structure, or both (Hall *et al.*, 1996).

The deception that *S. pneumoniae* employs to make itself unrecognisable to the immune system is to change its antigenic structure; this is achieved by some considerable genetic changes largely achieved by importing foreign DNA. As a result, there are 90 known different capsular serotypes together with their antigenic formulae (Henrichsen, 1995). Several groups have provided evidence that members of a single-resistant clone may express distinct and different capsular polysaccharides. Variants of a particular clone that are identical in overall genotype and in their antimicrobial resistance profile, but which are of different serotype, have been reported (Coffey *et al.*, 1991; Barnes *et al.*, 1995). The serotypes variants have been shown to have arisen, on multiple occasions, by large recombinational exchanges at the capsular biosynthetic locus (Coffey *et al.*, 1998). These results do question the validity of phenotypic typing systems and infer that they should

probably never be used in isolation but in combination with a genotypic typing system. If measurement of clonal spread is needed, then an consistent and stable parameter must be measured. Serotyping does not fall into this category.

9.4. Molecular Analysis of Penicillin Resistance and the Role of Clonal Spread

S. pneumoniae is one of the few bacterial species in which β-lactamases remain unknown and resistance to β-lactam antibiotics is entirely due to the development of altered PBPs that have greatly decreased affinity for penicillins and cephalosporins (Coffey *et al.*, 1995). Pneumococci possess five high molecular weight PBPs, but genetic studies have shown that high-level resistance to penicillin in clinical isolates results entirely from alterations of PBP1a, 2b and 2x (Barcus *et al.*, 1995), whereas high-level resistance to extended-spectrum cephalosporins derives from alterations of only PBP1a and 2x (Coffey *et al.*, 1995).

Several different approaches have been adopted for the molecular diagnosis of penicillin resistance. Ubukata *et al.* (1996) used a combination of PCRs that were targeted on *lytA*, a 240-bp fragment from the *pbp* susceptible strains, and two different penicillin mutant *pbp2b* gene sequences. Another group used primers based on susceptible *pbp2x* and *2b* genes and a resistant *pbp1a* gene (Jalal *et al.*, 1997). Their results show that all of these approaches are excellent at verifying fully susceptible strains. This is to be expected because of the genetic conservation of penicillin-susceptible genes (Dowson *et al.*, 1994). On the other hand, both fully PCR-based systems had difficulty detecting resistant isolates (Ubukata *et al.* 1996; Jalal *et al.*, 1997). All of the resistant isolates identified were truly resistant, but those strains with a sequence not encompassed by the primers were not amplified and thus gave false-negative results (Ubukata *et al.* 1996; Jalal *et al.*, 1997; O'Neill *et al.*, 1999). This problem is overcome by another PCR approach in which the

primer designed is based on a sequence which is conserved in susceptible and resistant strains (Gillespie *et al.*, 1997; Moissenet *et al.*, 1997; O'Neill *et al.*, 1999; Yoshida *et al.*, 1999). Thus, genes from susceptible and resistant isolates are amplified. It is then possible to determine resistance because the pattern of endonuclease digestion differs enough between different resistant alleles (O'Neill *et al.*, 1999).

In this thesis, two PCR protocols were employed to analyse the alteration in PBP2x, 2b and 1a in penicillin-resistant strains. Using the first PCR protocol, all the penicillin-resistant isolates were screened with primers to amplify susceptible genotypes of *pbp2x* and *2b* genes, and resistant genotype of *pbp1a* gene (Jalal *et al.*, 1997). The second PCR protocol involved the amplification of PBP2x, 2b and 1a whole genes followed by restriction of the PCR products using *Hin*fI restriction endonuclease (Gillespie *et al.*, 1997).

Screening for changes in PBPs showed that there was a correlation between resistance and alteration in PBP. All 10 strains with an MIC of penicillin of 2 mg/L or above had changes in *pbp1a* and all but one also had a change in *pbp2b*. In contrast only 6 had alterations in *pbp* 2x. However, isolates with MICs of penicillin less than 1 mg/L showed a more heterogeneous pattern of PBP changes and 2 isolates had no change in *pbp 1a, 2x* or *2b*. This approach also had difficulty in detecting resistant isolates, simply because the sequences of these strains are not recognised by the primers used in PCR and gave false negative results. To overcome this problem, a restriction digestion of the amplified PBP2x, 2b and 1a whole genes with *Hin*fI restriction endonuclease was performed. *S. pneumoniae* strains with an MIC of penicillin of 1 mg/L or above exhibited considerable homogeneity in *pbp2b* and *2x* RFLP. However, strains with an MIC less than 1 mg/L gave more heterogeneous patterns. It proved impossible to amplify *pbp1a* gene. This results is in agreement with recent published papers which concluded that RFLP of PBPs genes are likely to be one of the most useful tools for genetic analysis and for determining penicillin susceptibility in *S. pneumoniae* (O'Neill *et al.*, 1999; Yoshida *et al.*, 1999).

9.5. The Role of Fluoroquinolone Resistance in Limiting the Efficacy of These Drugs

Over the past two decades, the emergence and, in some areas, the prevalence of pneumococci with decreased susceptibility to penicillin have emphasised the need for new therapeutic agents and have focused attention on the fluoroquinolones. However, ciprofloxacin, the main quinolone in current clinical use, has modest activity against gram-positive bacteria such as *S. pneumoniae*. Although the rates of resistance of *S. pneumoniae* to ciprofloxacin are currently low, selection of resistance on exposure *in vitro* has been reported (Pan & Fisher, 1996; Pan *et al.*, 1996). Resistance to quinolone results from mutational alterations in the target sites: topoisomerase IV and DNA gyrase, coded for by the *parC* and *gyrA* genes respectively. Moreover, it has recently been observed that a third mechanism of ciprofloxacin resistance, in form of active efflux mechanism, exists in *S. pneumoniae* (Brenwald *et al.*, 1998).

In this study, the mechanisms of high-level ciprofloxacin resistance (MIC $\geq$ 2 mg/L) in *in vitro*-selected mutants and clinical isolates of penicillin-resistant *S. pneumoniae* have been examined. Characterisation of the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes by PCR, *Hin*fI restriction fragment length polymorphism (RFLP), and DNA sequence analysis revealed no apparent changes in *gyrA* and *parC* genes in clinical strains. Also no evidence of an efflux pump mechanism have been found. There may be nucleotide changes occurred outside the QRDRs of the *gyrA* or *parC* genes which not been amplified by the primers used in the current study. In contrast, *in vitro*-selected mutants from clinical strain, D-680, showed an 8-fold increase in ciprofloxacin MIC and corresponding to an increase for the new quinolones MICs from 2- to 16-fold. These mutants had acquired mutations in *gyrA* and *parC* QRDRs at Glu-87 to-Lys and at Ser-79 to-Tyr, respectively. These genetic changes are associated with increase in

ciprofloxacin and new quinolones MICs. The results show that mutants selected in the laboratory and those examined from clinical isolates may be quite distinct and it may be impossible to extrapolate information from *in vitro* studies to the clinical situation.

A recent study by Pan & Fisher (1998) suggested that there was dual targeting, of DNA gyrase and topoisomerase IV, by clinafloxacin against *S. pneumoniae*. They conclude this from examination of first and second step laboratory mutants in the emergence of bacterial resistance. The mutations selected by clinafloxacin challenge in their study were the *gyrA* mutations altered GyrA Glu-87 (to Gln or Lys), and the *parC* mutations resulted in ParC Ser-79 (to Phe or Tyr) and Asp-83 (to Ala) changes. They used this anecdotal information to conclude that the two targets were of equal importance. If mutations in clinical strains are quite unlike these laboratory-generated mutants, then the action of the fluoroquinolones may not be so easy to predict from mutation studies.

Correlations between the *in vitro* activity of ciprofloxacin against *S. pneumoniae* and its clinical efficacy are instructive. In a large study of both penicillin-susceptible and penicillin-resistant isolates, a mean MIC_{50} of 1.1 mg/L (range 0.5-2 mg/L) and a mean MIC_{90} of 2 mg/L (range 1-4 mg/L) were found in the two groups respectively (Kayser & Novak, 1987). These MICs are close to the current BSAC breakpoints for ciprofloxacin-susceptible *S. pneumoniae* (<2 mg/L). Although the rates of resistance of *S. pneumoniae* to ciprofloxacin are currently low, selection of resistance on exposure *in vitro* has been reported. Resistance results from single step mutations which lead to a stepwise increase in MICs and as ciprofloxacin MICs of *S. pneumoniae* already close to resistance breakpoint those organisms are much likely to become resistant.

9.6. The Influence of β-Lactam Resistance in the Spread of *H. influenzae*

β-Lactamases

The major mechanism of resistance to β-lactams drugs in *H. influenzae* is the production of β-lactamases, and the prevalence of lactamase-producing isolates has increased over the last 20 years (Powell *et al.*, 1987 & 1992; Barry *et al.*, 1994). Impermeability and alteration in PBPs are further mechanisms that mediate resistance to β-lactams (Reid *et al.*, 1987).

The most important β-lactamase in *H. influenzae* is TEM-1, which is found in more than 80% of β-lactamase-positive isolates (Scriver *et al.*, 1994). A second β-lactamase, ROB-1, has also been detected in *H. influenzae* (Robin *et al.*, 1981). Although less numerous than TEM-1, ROB-1 has been found in up to 8% of isolates in some studies (Daum *et al.*, 1988). Until recently, these were the only β-lactamases that had been described in *H. influenzae*; however, recent evidence suggests that the situation may be more complex. First, DNA sequence analysis of TEM-1 genes in *H. influenzae* suggested that the bla_{TEM-1} gene in this species may differ from its *E. coli* counterpart, and that silent amino-acid substitutions in TEM-1 have occurred (Vali *et al.*, 1995). Second, a completely novel β-lactamase, VAT-1, has also been identified recently in *H. influenzae* (Vali *et al.*, 1994). The significant of this enzyme is not clear at present, but it has been detected in isolates from different geographical centres in Scotland (Shanahan *et al.*, 1996; Vali *et al.*, 1995).

β-lactamases in *H. influenzae* confer significant resistance to β-lactam antibiotics. It is, therefore, essential that the production of β-lactamases can be identified in clinical isolates and that there effective ways of distinguishing the different enzymes. This is necessary for epidemiologic surveys, predicting future resistance trends, and to ensure

that patients receive the appropriate β-lactam or alternative therapy. The first indication of the presence of β-lactamases is usually the observation of increased levels of resistance in clinical isolates as detected by routine susceptibility testing. Analytical isoelectric focusing (IEF) can then be used to characterised the β-lactamase produced. Molecular techniques have become very effective for identifying different β-lactamases (Payne & Thomson, 1998). PCR has been used to detect ampicillin resistance genes in CSF samples containing *H. influenzae* (Tenover *et al.*, 1994). Correlation was obtained between the results of MIC testing, β-lactamase production as determined by nitrocephin and PCR testing (Tenover *et al.*, 1994). Molecular typing by pulsed-field gel electrophoresis has also been used to assess clonal relationship of isolates (Gazagne *et al.*, 1998; Moor *et al.*, 1999).

Molecular Typing

In this study, the epidemiology and mechanisms of resistance to amoxycillin in *H. influenzae* isolates was examined. Isolates were genotyped by pulsed-field gel electrophoresis (PFGE), and screened for β-lactamase production by isoelectric focusing (IEF) followed by PCR employing primers for the TEM-1 β-lactamase. IEF demonstrate that all the β-lactamase-positive strains produced an enzyme that co-focused with TEM-1. PCR employing a universal TEM-1 primer confirmed identity of the β-lactamase. Cluster analysis of patterns produced by PFGE of chromosomal DNA digested with *Sma*I revealed that amoxycillin-resistant isolates showed little genetic relatedness. However, few clusters of isolates have been identified. According to these results, it seems that the clonal propagation of these resistant strains does not occur as previously described *in S. pneumoniae* clinical isolates (Muñoz *et al.*, 1991). It suggests that the $bla_{\text{TEM-1}}$ gene is located on a mobile genetic element, such as a plasmid, as it is in large gram-negative rods. In this study, amoxycillin resistance in *H. influenzae* throughout the UK is

predominantly mediated by the TEM-1 enzyme which appears to have disseminated into a wide variety of different strains.

Non-Enzymic Mechanisms of Resistance

β-Lactam resistance in *H. influenzae* is mainly mediated by the production of β-lactamases. The extent of non-β-lactamase-mediated resistance has always been difficult to assess and it is possible that β-lactamase production may be missed by routine testing (Scriver *et al.*, 1994). β-lactam resistance in strains apparently negative for β-lactamase production has been attributed to target modification (PBP alterations) and/or impermeability (OMP alterations) (Reid *et al.*, 1987). However, some of this evidence was circumstantial.

In the current study, impermeability and modification of PBPs were demonstrated in few co-amoxiclav-resistant isolates. Comparison of the OMPs profiles revealed that similar patterns were observed in most of the co-amoxiclav-resistant strains compared with the susceptible strain. However, two strains, RIE-233 and FL-258, lacked the 39.8 KDa band which was present in all the other strains. Interestingly, these two isolates have the same MIC results for amoxycillin and co-amoxiclav (2 mg/L, respectively), non-β-lactamase producer, and gave similar PFGE patterns. Both isolates were from different geographical location. In contrast, the 9 co-amoxiclav-resistant strains tested exhibited marked heterogenicity in PBPs patterns. As there was variations in electrophoretic mobility as well as in the amount of penicillin bound by certain PBPs compared with susceptible control strain. Also the PBPs of certain isolates was either markedly reduced or absent.

Doern *et al.*, (1997) also demonstrated that non-β-lactamase resistance to amoxycillin also confered resistance to co-amoxiclav. They concluded that this could cause problems for treatment but would also require close interpretation of diagnostic sensitivity tests.

The increase in this type of resistance would certain cause significant treatment difficulties. This study has established that β-lactamase enzymes are not the sole mechanism of amoxycillin resistance in the *H. influenzae* strains and resistance may also result from PBP and OMP alterations. However, the introduction of the non-enzymic resistance mechanisms does broaden the spectrum of resistance to embrace β-lactamase inhibitor combinations and some cephalosporins such as cefaclor.

Concluding Remarks

Treatment of community-acquired respiratory infections often starts empirically. Thus, it is important to know which organism are most likely to occur, the usual resistance patterns, and which is the best group of antibiotics to use. The choice of appropriate antibiotics should take into account not only the interests of the individual patient, but also the ecological impact of different drugs and their delivery schedules. Avoidance of selection of antibiotic-resistant organisms is a key aspect to remember.

It is indisputable that aminopenicillins promote the carriage of penicillin-resistant pneumococci (Baquero, 1996). In common with other β-lactams, they may select penicillin-resistant strains already present in the oropharynx or other penicillin-resistant oral streptococci which might serve as reservoirs for resistance genes that encode mutations leading to alterations to the PBPs of pneumococci. However, because of cross-resistance and reduced activities against penicillin-resistant pneumococci, the selective pressures exerted by oral cephalosporins are even greater. The selection of penicillin-resistant pneumococci by oral cephalosporins, both *in vitro* and *in vivo*, can be explained by their reduced activity against penicillin-resistant strains, as well as by the fact that the modification of a single PBP, PBP 2x, may result in marked increase in MICs (Coffey *et al.*, 1995; Hakenbeck, 1999). Concurrent resistance to other antibiotics, including macrolides and co-trimoxazole, in multidrug-resistant strains will not only select still

greater numbers of resistant clones in the nasopharynx, but may also lead to clinical failures, thereby increasing the risk of disseminating penicillin-resistant pneumococci (Arason *et al.*, 1996).

Over 20 years children have received oral ampicillin or amoxycillin for the treatment of *H. influenzae* meningitis of which a significant percentage must have been caused by β-lactamase-producing strains. The clinical relevance of β-lactamase production by *H. influenzae* for treatment failure in meningitis is clearly established (Needham, 1988). While it is intuitive to believe that β-lactamase production by *H. influenzae* may lead to treatment failure in the management of pneumonia, there is little confirmation of this in the literature. The *in vitro* identification of ampicillin resistance due to β-lactamase production depends upon a sufficient inoculum of organisms, usually $>10^5$/ml (Needham, 1988). While this concentration of organisms is regularly achieved in the CSF of children with *H. influenzae* meningitis (Feldman, 1976), it is possible that lower numbers of organisms may be susceptible *in vivo* to ampicillin even though they produce β-lactamase. The breakpoint for *H. influenzae* resistance to β-lactams of 1 mg/L was defined on the basis of laboratory criteria (Philips *et al.*, 1991) and is much lower than the achievable blood concentrations. As the concentration of organisms in blood is very low, it is probable that amoxycillin therapy would be successful.

Erythromycin has little activity against *H. influenzae*. However, data demonstrating azithromycin activity is currently being accumulated (Retsema *et al.*, 1987; Doern *et al.*, 1997). Resistance to tetracycline and trimethoprim has been detected, but is currently low (Brown *et al.*, 1996). Although there are a number of isolated reports of the emergence of quinolone resistance in *H. influenzae* (Gould *et al.*, 1994), this does not appear to be a widespread phenomenon and resistance to ciprofloxacin and other quinolones remain low.

Final Statement

The results of this thesis show that aminopenicillins select both resistant *S. pneumoniae* and *H. influenzae*. Also it shows that the use of amoxycillin plus clavulanic acid has little impact on the development of resistance in *H. influenzae* but may have a great impact on the selection of penicillin resistant pneumococci. As new therapies are considered, the new fluoroquinolones must be prime candidates. This thesis suggests that resistance in *H. influenzae* is effectively non-existent despite the fact that ciprofloxacin has been used for 12 years and also suggests that the impact on the control of *S. pneumoniae* will be substantial. Even though these bacteria have been exposed to ciprofloxacin for more than a decade, there is no evidence for cross-resistance to newer fluoroquinolones.

My results demonstrate that the increased resistance in *S. pneumoniae* and *H. influenzae* may affect treatment options with traditional penicillin-containing therapy. They suggest that amoxycillin plus clavulanic acid probably remains the best therapy in susceptible populations but if they become resistant, the newer fluoroquinolones stand a very good chance in empirical therapy. It looks as though the newer macrolides are not a good general therapy. If these fail particularly against Gram-positive bacteria, we should probably have to consider combination therapy with one antibiotic, such as a fluoroquinolone, to control the Gram-negative pathogens and a glycopeptide to control *S. pneumoniae*. We hope that judicious use of the fluoroquinolones as a supplement to β-lactam therapy may make this scenario unnecessary.

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